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Stress tolerance of *Listeria monocytogenes* and control of the bacterium in the fish industry

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DOCTORAL DISSERTATION

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Food safety does not happen by accident

Christopher James Griffith, 2010

ABSTRACT

The severe foodborne disease listeriosis is caused by the bacterium *Listeria monocytogenes*, known as a problematic contaminant of the food chain. This facultative anerobe tolerates many conditions used for controlling harmful bacteria, including high salinity and temperature. Some *L. monocytogenes* strains tolerate external stressors better than others, which may complicate the control of the bacterium in food-related environments. Strains exposed to one stress condition may also develop tolerance towards another; such cross-adaptation occurs, for instance, between osmotic and heat stress. In food production, *L. monocytogenes* may encounter these stresses via salting and heat treatments or hot water used in sanitation. Vacuum-packaged ready-to-eat fish products frequently contain *L. monocytogenes* and have caused several listeriosis outbreaks. They often do not undergo listericidal processes before consumption, and thus, their processing requires stringent preventive measures. The aims of this dissertation were to investigate the strain variability and determinants of *L. monocytogenes* stress tolerance and to examine the framework of fish-processing plants and their official food control for managing *L. monocytogenes* contamination.

Using optical density measurements of microbial growth, differences in growth ability under osmotic (NaCl) stress were determined for 388 wild-type *L. monocytogenes* strains. Notable strain variability as well as serotype- and lineage-dependent patterns of *L. monocytogenes* salt stress tolerance were discovered. Lineage-I-affiliated *L. monocytogenes* serotype 1/2b and 4b strains grew significantly better at NaCl 9.0% than lineage-II-affiliated serotypes 1/2a, 1/2c, and 3a. By enabling this comprehensive identification of NaCl-tolerant strains, our data assembly and analysis protocol elucidated the biologically relevant intra-species variability of *L. monocytogenes* salt stress tolerance phenotypes.

A comparative whole-genome sequencing approach was implemented to identify underlying determinants of *L. monocytogenes* stress tolerance phenotypes. Accessory genetic mechanisms of stress resistance were investigated by comparison of heat survival phenotypes and whole-genome sequences of a heat-resistant and a heat-sensitive *L. monocytogenes* strain. The comparison identified a novel plasmid, pLM58, including an open reading frame annotated as an adenosine triphosphate (ATP) -dependent ClpL-protease-encoding gene, which was present in the heat-resistant strain but absent in the heat-sensitive strain. The curing of pLM58 resulted in a reduction of heat resistance. The conjugation of *clpL* increased the heat survival of a natively heat-sensitive *L. monocytogenes* strain. This study described, for the first time, plasmid-borne heat resistance of *L. monocytogenes* and identified the protease ClpL as a novel mechanism of *L. monocytogenes* heat resistance.

To examine the framework for managing *L. monocytogenes* contamination in the fish industry, operational practices and efficacy of official control were studied in 21 Finnish fish-processing plants producing vacuum-packaged gravad (cold-salted) and cold-smoked fish products. Product samples were investigated for the presence and quantity of *L. monocytogenes* in 2014–2015. Additionally, the results of official food control sampling of products and facilities were assessed to retrospectively gain information on *L. monocytogenes* contamination in the participating fish-processing plant facilities in 2011–2013. The production and hygiene practices of the processing plants were surveyed with an in-depth inspection questionnaire, and the occurrence, control measures, and correction of non-compliances were drawn from their official inspection records. Associations of *L. monocytogenes* occurrence with fish-processing plant operational practices, compliance, and aspects of official control during the respective years were investigated with statistical modeling.

L. monocytogenes product contamination was associated with number of processing machines, deficiencies in the processing environment and machinery sanitation, and staff movement from areas of low hygiene to high hygiene. Performing frequent periodic thorough sanitation was associated with a decreased risk of product contamination. The increased occurrence of *L. monocytogenes* in the facilities and products of the fish-processing plants was associated with hygiene deficiencies in processing machinery, a lack of demanding control measures for non-compliances, and recurrence of non-compliances. These results identified areas for improvement in the preventive measures of fish-processing plants and official food control, providing ways to reduce *L. monocytogenes* contamination in the fish industry.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles which are referred to in the text by their Roman numerals:

I Aalto-Araneda, M., Pöntinen, A., Pesonen, M., Corander, J., Markkula, A., Tasara, T., Stephan, R. & Korkeala, H. 2020. Strain variability of *Listeria monocytogenes* under NaCl stress elucidated by a high-throughput microbial growth data assembly and analysis protocol. *Applied and Environmental Microbiology*, 86, e02378-19.

II Pöntinen, A., Aalto-Araneda, M., Lindström, M. & Korkeala, H. 2017. Heat resistance mediated by pLM58 plasmid-borne ClpL in *Listeria monocytogenes*. *mSphere* 2, e00364–17.

III Aalto-Araneda, M., Lundén, J., Markkula, A., Hakola, S. & Korkeala, H. 2019. Processing plant and machinery sanitation and hygiene practices associate with *Listeria monocytogenes* occurrence in ready-to-eat fish products. *Food Microbiology* 82, 455–464.

IV Aalto-Araneda, M., Korkeala, H. & Lundén, J. 2018. Strengthening the efficacy of official food control improves *Listeria monocytogenes* prevention in fish-processing plants. *Scientific Reports* 8, 13105.

ABBREVIATIONS

AIC	Akaike information criterion
ALOA	Harlequin *Listeria* chromogenic agar (Ottavani & Agosti)
ATP	Adenosine triphosphate
AUC	Area under the curve
a_w	Water activity
BHI	Brain-heart infusion
BHIB	Brain-heart infusion broth
β	Likelihood ratio estimate
cfu	Colony-forming unit
CI	Confidence interval
CV	Coefficent of variation
DALY	Disability-adjusted life year
Df	Degree of freedom
DNA	Deoxyribonucleic acid
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EU	European Union
HACCP	Hazard Analysis and Critical Control Points
IBM	International Business Machines Corporation
ISO	International Organization for Standardization
LB	Luria-Bertani (lysogeny broth)
λ	Lag time
MaxOD	Maximum optical density
MLST	Multilocus sequence typing
μ	Maximum specific growth rate
NaCl	Sodium chloride
OD_{600}	Optical density at 600 nm
OR	Odds ratio
ORF	Open reading frame
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
R	R computational environment
RNA	Ribonucleic acid
RTE	Ready-to-eat
SE	Standard error
ST	Sequence type
TSA	Tryptic soy agar
TSB	Tryptic soy broth

1 INTRODUCTION

Listeria monocytogenes is the causative agent of the zoonotic and foodborne bacterial disease listeriosis. The bacterium is believed to have been first isolated from a necrotic rabbit liver and given the name *Bacillus hepatis* in the early 1900s (Hülphers, 1911). The first established description by the name of *Bacterium monocytogenes* was published as a discovery of a disease characterized by monocytosis in rabbits (Murray *et al.*, 1926). Concurrently, the bacterium was described in a novel rodent disease and named *Listerella hepatolytica* (Pirie, 1927). Since 1940, it has been known as *Listeria monocytogenes* (Pirie, 1940). In the years following its identification, *L. monocytogenes* was on rare occasions reported in human illness (Nyfeldt, 1929). Early cases of human listeriosis may also have been misidentified (Seeliger, 1988), as was the first isolate preserved from human meningitis (Dumont & Cotoni, 1921).

Feed was suspected as a major cause of listeriosis in domestic ruminants (Gray & Killinger, 1966), and similarly, food was assumed to be a source of human listeriosis in the 1950s (Seeliger, 1988). Foodborne transmission of *L. monocytogenes* was confirmed only after several human outbreaks occurred in the 1980s (Schlech *et al.*, 1983; Fleming *et al.*, 1985; Linnan *et al.*, 1988). Notably, the emergence of foodborne listeriosis coincides with the development of modern food production. The contemporary chilled food chain has brought about long product shelf lives and complex processing environments, providing favorable settings for the survival, growth, and persistence of *L. monocytogenes* (Linnan *et al.*, 1988; Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Dauphin *et al.*, 2001; Norton *et al.*, 2001a). Nevertheless, given a noteworthy publication bias, the burden of listeriosis in many regions of the world is still largely unknown (Ababouch, 2000; Destro, 2000; de Noordhout *et al.*, 2014; Paudyal *et al.*, 2017; Hamidiyan *et al.*, 2018).

With an estimated 23 000 cases and 170 000 disability-adjusted life years (DALYs), i.e., years of healthy life lost, worldwide in 2010 (de Noordhout *et al.*, 2014), the overall burden of human listeriosis is relatively low. Nonetheless, the high case fatality rate of approximately 20% (de Noordhout *et al.*, 2014; Desai *et al.*, 2019) and the increase of invasive listeriosis in Europe in the 2010s (European Food Safety Authority, EFSA, & European Centre for Disease Prevention and Control, ECDC, 2018) constitute a growing public health concern. In Finland, the annual average of human listeriosis cases has grown from 34 in 2000–2009 to 64 in 2010–2019 i.e., from 0.64 to 1.2 cases per 100 000 inhabitants, respectively (National Institute for Health and Welfare, 2019; Official Statistics of Finland, 2019). The rising incidence can be partly attributed to increasing susceptible populations – namely the elderly and immunocompromised – as modern medicine is progressively able to

prolong life and manage chronic diseases (Goulet *et al.*, 2008; Ricci *et al.*, 2018). Listeriosis outbreaks also raise concern over the supply of foodstuffs contaminated with *L. monocytogenes* (Lopez-Valladares *et al.*, 2018; Ricci *et al.*, 2018).

Zoonotic infections often emerge from complex interactions between ecological and societal processes (Waltner-Toews, 2017), and listeriosis is no exception. *L. monocytogenes* transitions between saprophytic and pathogenic lifestyles (Chaturongakul *et al.*, 2008), inhabiting natural and built environments, farmed lands, and various animal hosts. The entire food chain – from primary production, food processing, and retail facilities to consumer homes – serves as a habitat for *L. monocytogenes*. To avoid the contamination, survival, and growth of *L. monocytogenes* in the food chain, various factors concerning the characteristics of the bacterium, production systems, and preventive actions must be understood. Holistic understanding can be achieved by combined research efforts examining biological, environmental, and social drivers of the disease, known as the One Health approach (Gibbs, 2014).

Tolerance towards various external conditions is important for the virulence of *L. monocytogenes* (Gahan & Hill, 2005; Chaturongakul *et al.*, 2008; de las Heras *et al.*, 2011) and its endurance in food-related environments (NicAogáin & O'Byrne, 2016; Bucur *et al.*, 2018). Traditional means of preventing bacterial growth are largely inefficient against *L. monocytogenes*, as it tolerates a wide array of conditions encountered in the food chain, including cold, heat, and osmotic stress (Markkula *et al.*, 2012a; Markkula *et al.*, 2012b). Recognizing which *L. monocytogenes* strains tolerate stressors better than others enables the investigation of their underlying accessory stress tolerance mechanisms. From the perspective of the food chain, this facilitates the identification of potentially problematic strains and their determinants. Presently, increasing capacity to produce high-throughput research datasets and utilize next-generation sequencing opens new horizons to identify variability and genetic mechanisms of bacterial phenotypes (Van Der Veen *et al.*, 2008; Moura *et al.*, 2016).

L. monocytogenes adheres to surfaces (Spurlock & Zottola, 1991; Norwood & Gilmour, 1999; Lundén *et al.*, 2000) and is difficult to remove from processing facilities by perfunctory sanitation (Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Lappi *et al.*, 2004b). The oftentimes stringent sanitation measures and structural renovations required for *L. monocytogenes* control (Lundén *et al.*, 2002; Lappi *et al.*, 2004b; Keto-Timonen *et al.*, 2007) place labor and financial burden on food industry operators. Several listeriosis outbreaks have been connected to poor hygiene in fish-processing plants (Tham *et al.*, 2000; Nakari *et al.*, 2014; Gillesberg Lassen *et al.*, 2016; ECDC & EFSA, 2019). While product safety is the producers' responsibility, food control authorities guide and oversee compliance with food safety legislation. As food safety actions are powered by human hands, attitudes, and motivations (Yapp & Fairman, 2006; Griffith, 2010), the efficacy of preventive measures and official food control in

fish-processing plants warrants investigation. All in all, combining phenotypic and mechanistic perspectives of stress tolerance with insights into food safety management and official food control broadens the understanding of the ecology and epidemiology of the foodborne pathogen *L. monocytogenes*.

2 REVIEW OF THE LITERATURE

2.1 *L. monocytogenes* in humans, other animals, and the environment

The Gram-positive, non-spore-forming, psychrotrophic, halotolerant, facultatively anaerobic saprophyte and opportunistic intracellular pathogen *L. monocytogenes* leads a flexible lifestyle within several environments, including soil, water, sewage, silage, food, and primarily mammalian hosts (Gray & Killinger, 1966; Weis & Seeliger, 1975; McLauchlin & Rees, 2015). While food and feed act as vechicles between its environmental and pathogenic lifestyles (Wilesmith & Gitter, 1986; Farber & Losos, 1988; Vázquez-Boland *et al.*, 1992), asymptomatic carriage and fecal shedding of *L. monocytogenes* have been reported in wild and domestic animals, including humans (Weis & Seeliger, 1975; Lamont & Postlethwaite, 1986; Husu, 1990; Miettinen *et al.*, 1990; Husu *et al.*, 1990a; Grif *et al.*, 2001; Nightingale *et al.*, 2004; Lyautey *et al.*, 2007b; Hellström *et al.*, 2008; Stea *et al.*, 2015).

The size of the *L. monocytogenes* chromosome is approximately 2.9 million base pairs consisting of roughly 2800 protein-coding genes and an average G+C content of 39% (Glaser *et al.*, 2001). *L. monocytogenes* is presently divided into four genetic lineages (Orsi *et al.*, 2011; Haase *et al.*, 2014; Moura *et al.*, 2016), the subtypes in which can be categorized by various phenotyping and molecular genotyping methods (Nightingale, 2010). Serotyping (Seeliger & Höhne, 1979; Doumith *et al.*, 2004) and the more recent multilocus sequence typing (MLST) (Salcedo *et al.*, 2003; Haase *et al.*, 2014; Moura *et al.*, 2016) are usually referenced when characterizing the presence of *L. monocytogenes* subtypes in different hosts and environments. Genome-wide analyses have established that lineage II consists of a more diverse and recombinant population of *L. moncytogenes* isolates than the highly clonal lineage I (den Bakker *et al.*, 2008; Orsi *et al.*, 2008), the isolates of which appear to inhabit a less diverse range of environments than lineage II isolates (Orsi *et al.*, 2008).

2.1.1 Listeriosis

Of the 20 currently described *Listeria* spp. (Murray *et al.*, 1926; Pirie, 1940; Larsen & Seeliger, 1966; Rocourt & Grimont, 1983; Seeliger, 1984; Rocourt *et al.*, 1992; Graves *et al.*, 2010; Leclercq *et al.*, 2010; Bertsch *et al.*, 2013; Lang Halter *et al.*, 2013; den Bakker *et al.*, 2014; Weller *et al.*, 2015; Doijad *et al.*, 2018; Núñez-Montero *et al.*, 2018; Leclercq *et al.*, 2019), *L. monocytogenes* is the principal causative agent of human listeriosis (Gray & Killinger, 1966; Farber & Losos, 1988; Vázquez-Boland *et al.*, 2001a; McLauchlin & Rees, 2015). Rare human cases by *Listeria ivanovii*, *Listeria innocua*, and *Listeria*

grayi have been reported (Cummins *et al.*, 1994; Perrin *et al.*, 2003; Rapose *et al.*, 2008). The pathogenic potential of atypical hemolytic *L. innocua* strains has recently been characterized (Moura *et al.*, 2019).

L. monocytogenes lineage I serotypes 4b and 1/2b have often been linked to human cases, and lineage III isolates to other animals, while serotypes 1/2a, 1/2c, and 3a belonging to genetic lineage II have typically been isolated from foods (Orsi *et al.*, 2011; Paduro *et al.*, 2020). Lineage II *L. monocytogenes* isolates have caused human listeriosis in the Nordic countries and progressively around Europe and North America (Lukinmaa *et al.*, 2003; Parihar *et al.*, 2008; Lopez-Valladares *et al.*, 2018). The occurrence of *L. monocytogenes* serotype 1/2a in increasingly popular ready-to-eat (RTE) foods might partially explain its emerging coincidence with human listeriosis (Lopez-Valladares *et al.*, 2018).

Human listeriosis is predominantly a foodborne disease (Mead *et al.*, 1999; Ricci *et al.*, 2018). After ingestion, *L. monocytogenes* is exposed to acidity in the stomach and bile acids and salts in the small intestine (Schlech *et al.*, 1993; Dussurget *et al.*, 2002; Sleator *et al.*, 2005; Watson *et al.*, 2009; Payne *et al.*, 2013). The cells that survive pass through the intestinal mucosa, where *L. monocytogenes* infects non-phagocytic cells and macrophages and proliferates inside them, shielded from the extracellular environment (Vázquez-Boland *et al.*, 2001b; Radoshevich & Cossart, 2017). In macrophages, *L. monocytogenes* can escape the phagosome (Henry *et al.*, 2006) or stop its maturation into an oxidatively and enzymatically degrading phagolysosome and proliferate within phagosomal vacuoles (Birmingham *et al.*, 2008). Entering the hepatic circulation and lymph, *L. monocytogenes* reaches the liver and spleen, where it is destroyed by Kuppfer cells and T-cells (Ebe *et al.*, 1999; Gregory & Liu, 2000). If the host's cell-mediated immunity is impaired, *L. monocytogenes* may enter the general circulation and pass through the blood-brain and fetoplacental barriers, causing invasive disease (Gregory & Liu, 2000; Vázquez-Boland *et al.*, 2001b; Radoshevich & Cossart, 2017).

In humans, listeriosis may exhibit as a mild to severe febrile gastroenteritis in otherwise healthy adults after the ingestion of highly contaminated foodstuffs (Dalton *et al.*, 1997; Miettinen *et al.*, 1999b). However, human listeriosis is better known for its systemic form, which has a case fatality rate of 15–26% (de Noordhout *et al.*, 2014). Among people at risk, including neonates, the elderly, and immunocompromised individuals, this severe invasive illness manifests as septicemia, meningitis, encephalitis, and perinatal infections, and causes abortions among pregnant women (Vázquez-Boland *et al.*, 2001b; de Noordhout *et al.*, 2014). Outside these risk groups, listerial meningitis in otherwise healthy children aged 5–15 years has recently been reported (Angelo *et al.*, 2017). Invasive listeriosis is presumed to typically result from ingestion of large doses of *L. monocytogenes*, but the infectious dose may vary according to individual susceptibility (World Health Organization & Food and Agriculture Organization of the United Nations,

2004; Buchanan *et al.*, 2017), and relatively low levels of *L. monocytogenes* contamination have also been present in some implicated foodstuffs (Ericsson *et al.*, 1997; Maijala *et al.*, 2001; Pouillot *et al.*, 2016). An estimated 90% of invasive listeriosis cases in Europe are presumed to be caused by the consumption of RTE foods with *L. monocytogenes* quantities of >2000 cfu/g (Ricci *et al.*, 2018).

In other animals, listeriosis predominantly manifests as an invasive disease involving septicemia, meningoecephalitis, and abortions (Low & Renton, 1985; Campero *et al.*, 2002; Lecuit, 2007), primarily affecting small ruminants and, to some extent, cattle (Nightingale *et al.*, 2004; Lecuit, 2007). Listeriosis can affect various species of mammals, birds, fish, reptiles, and amphibians (Lecuit, 2007). Both *L. monocytogenes* and *L. ivanovii* are pathogenic in domestic ruminants (Wilesmith & Gitter, 1986; Sergeant *et al.*, 1991; Alexander *et al.*, 1992; Chand & Sadana, 1999; Campero *et al.*, 2002; Nightingale *et al.*, 2004; Lecuit, 2007). Virulence of *Listeria* spp. against fish has been described (Menudier *et al.*, 1996; Hardi *et al.*, 2018); although *L. monocytogenes* appears not to multiply well in zebrafish (Menudier *et al.*, 1996), it is pathogenic to the larvae of this aquatic model organism used in bacterial virulence research (Levraud *et al.*, 2009; Vincent *et al.*, 2016).

2.1.2 Environmental spread of *L. monocytogenes*

Natural, farmed, and urban soil environments and plants contain *L. monocytogenes* (Welshimer & Donker-Voet, 1971; Weis & Seeliger, 1975; Sauders *et al.*, 2012). *L. monocytogenes* also appears in fresh, estuarine, and marine waters and sediments (Colburn *et al.*, 1990; Motes, 1991; Lyautey *et al.*, 2007a; Stea *et al.*, 2015). Accordingly, the environmental-oral-fecal spread of *L. monocytogenes* has been deduced to occur via circulation of the bacterium between soil, water, plants, and animals (Gray & Killinger, 1966; Vivant *et al.*, 2013).

The occurrence of *L. monocytogenes* in natural environments appears to be linked to agriculture and other anthropogenic influences (Colburn *et al.*, 1990; Motes, 1991; Ben Embarek, 1994; Gram, 2001; Miettinen & Wirtanen, 2006; Lyautey *et al.*, 2007a; Lyautey *et al.*, 2007b; Sauders *et al.*, 2012; Stea *et al.*, 2015). At dairy farms, contaminated silage and poor hygiene contribute to fecal shedding of *L. monocytogenes* by cattle (Husu, 1990; Husu *et al.*, 1990a; Husu *et al.*, 1990b; Sanaa *et al.*, 1993; Nightingale *et al.*, 2004; Castro *et al.*, 2018). Proximity to farm animals, dairy farms, and farmed land has been reported to be associated with the presence of *L. monocytogenes* and the abundance of its particular subtypes in estuarine and river waters (Colburn *et al.*, 1990; Lyautey *et al.*, 2007a). Moreover, similar *L. monocytogenes* isolates have been found from river waters and feces of dairy cattle, wildlife, and humans (Lyautey *et al.*, 2007b). Effluents of sewage treatment plants and animal processing facilities also contain *L. monocytogenes* (Watkins & Sleath, 1981; Motes, 1991). Correspondingly, contaminated water may transfer *L.*

monocytogenes to irrigated crops (Steele & Odumeru, 2004) and aquatic food production facilities (Miettinen & Wirtanen, 2006).

2.2 Stress tolerance of *L. monocytogenes*

In optimal conditions, which vary by bacterial species, bacterial growth and metabolism occur rapidly. By deviating from optimal conditions, stressors induce alterations in cellular structure and metabolism, requiring mitigative responses from the bacterial cell to sustain functionality (Soni *et al.*, 2011). Under mild stress, this redirection of metabolism results in cellular acclimation to the new conditions displaying as reduced growth such as increased delay in the initiation of multiplication (increased lag phase), decreased rate of multiplication during the exponential growth phase (decreased growth rate), and reduced population size reached by the stationary phase of growth (reduced maximum growth level). By contrast, severe stresses cause an immediate shock response and can damage the bacterial cell enough to kill it.

Stress tolerance and resistance, i.e., acclimation and endurance towards suboptimal and lethal environmental stress conditions, are vital to the survival and persistence of *L. monocytogenes* in the food chain (NicAogáin & O'Byrne, 2016; Bucur *et al.*, 2018) and the transition to its pathogenic lifestyle inside host organisms (Gahan & Hill, 2005; Chaturongakul *et al.*, 2008; de las Heras *et al.*, 2011). *L. monocytogenes* utilizes glycolysis, the pentose phosphate pathway, an incomplete citric acid cycle, and carbohydrate fermentation for its metabolism and can, hence, grow under aerobic and anaerobic conditions (Trivett & Meyer, 1971; Pine *et al.*, 1989; Romick *et al.*, 1996; Glaser *et al.*, 2001; Jydegaard-Axelsen *et al.*, 2004; Wallace *et al.*, 2017). The bacterium also grows over an extensive temperature and pH range (–1.5–45°C; pH 4.3–9.6) and in salinity of up to 10–11% (Gray & Killinger, 1966; Junttila *et al.*, 1988; Hudson *et al.*, 1994; Ribeiro & Destro, 2014).

As *L. monocytogenes* is highly tolerant of conditions traditionally used for controlling the growth of undesired bacteria during food production and storage, such as osmolality, temperature, and pH (Doyle *et al.*, 2001; Cotter & Hill, 2003; Tasara & Stephan, 2006; Chan & Wiedmann, 2009; Sergelidis & Abrahim, 2009; Burgess *et al.*, 2016; NicAogáin & O'Byrne, 2016; Bucur *et al.*, 2018), factors conveying *L. monocytogenes* stress tolerance have become an abundant – yet not exhaustive – field of study. The ability to grow under different external conditions has also been indicated to vary between *L. monocytogenes* strains (Faleiro *et al.*, 2003; Lianou *et al.*, 2006; Lundén *et al.*, 2008; Van Der Veen *et al.*, 2008; Bergholz *et al.*, 2010; Ribeiro & Destro, 2014). As exceptionally tolerant strains may pose a marked threat to food safety (Pouillot *et al.*, 2007), it is crucial to strive towards comprehensive understanding of the determinants and strain variability of *L. monocytogenes* stress tolerance.

2.2.1 Responses to stressors in the food chain

Solute addition and dessication decrease water activity (a_w) in the external environment, e.g., food matrix, which results in an osmotic gradient out of the bacterial cytoplasm towards the surroundings. Thereby, osmotic stress causes dehydration, loss of cell turgor and volume, and disruption of protein structure and function, leading to interferences in many vital functions of the bacterial cell (Soni *et al.*, 2011; Burgess *et al.*, 2016). *L. monocytogenes* faces osmotic conditions at several phases of the bacterial ecology: in marine environments (Colburn *et al.*, 1990; Motes, 1991; NicAogáin & O'Byrne, 2016), in mammalian hosts via osmolality and bile salts of the intestines and gall bladder (Watson *et al.*, 2009; Payne *et al.*, 2013), and in foodstuffs through additives, such as salt (NaCl), used for controlling bacterial growth and product flavor (Cornu *et al.*, 2006; Hwang *et al.*, 2009). Growth of *L. monocytogenes* has been reported in NaCl concentrations of up to 11% (Ribeiro & Destro, 2014), which would render foodstuffs organoleptically inedible. *L. monocytogenes* withstands osmotic stress by accumulating compatible solutes, such as glycine betaine and carnitine, to reduce osmotic pressure and stabilize enzymes (Fraser *et al.*, 2000; Duché *et al.*, 2002a; Duché *et al.*, 2002b; Angelidis & Smith, 2003b). Other mechanisms of *L. monocytogenes* osmotolerance include cell envelope modifications and DNA-RNA-protein metabolism (Kallipolitis & Ingmer, 2001; Wonderling *et al.*, 2004; Markkula *et al.*, 2012b; Burgess *et al.*, 2016).

High temperature denaturates proteins, degrades DNA and RNA, and damages the cytoplasmic membrane, leading to malfunction of bacterial enzymes and leakage of cellular components (Sergelidis & Abrahim, 2009; Soni *et al.*, 2011). *L. monocytogenes* encounters heat stress in the food chain when food products undergo heat treatments or warm water is used for the sanitation of facilities. *L. monocytogenes* tolerates heat treatments milder than pasteurization better than many other non-spore-forming foodborne pathogens (Doyle *et al.*, 2001). The optimal temperature for *L. monocytogenes* is 30–37°C, while growth ends at 45°C and destruction has been reported at temperatures of 60–83°C (Farber & Brown, 1990; Poysky *et al.*, 1997; McLauchlin & Rees, 2015). The response to heat stress by *L. monocytogenes* includes restoration of membrane, nucleic acid, and protein functions (Van Der Veen *et al.*, 2009; Soni *et al.*, 2011). Following heat stress, a specific set of heat shock proteins acting as chaperones aids in the degradation and folding of damaged proteins (Hanawa *et al.*, 2000; Nair *et al.*, 2000; van der Veen *et al.*, 2007; Hu *et al.*, 2007a; Hu *et al.*, 2007b).

In cold stress, the bacterial metabolism and transport slow down, fluidity of membranes decreases, cellular structures rigidify, and protein damage ensues (Tasara & Stephan, 2006; Chan & Wiedmann, 2009; Soni *et al.*, 2011). The modern food chain depends upon cold storage for the prevention of bacterial growth, preservation of foods, and elongation of shelf lives. However, *L. monocytogenes* is not disadvantaged by cold conditions; while under refrigeration temperatures, growth takes several days (Markkula *et al.*, 2012a;

Pöntinen *et al.*, 2015), *L. monocytogenes* has been reported to grow until slightly below 0°C (Gray & Killinger, 1966; Junttila *et al.*, 1988; Hudson *et al.*, 1994). *L. monocytogenes* mitigates the effects of cold stress by supporting membrane fluidity and transporting compatible solutes (Angelidis & Smith, 2003a; Tasara & Stephan, 2006; Chan & Wiedmann, 2009). Specific cold-shock proteins – some of which also play a role in osmotic stress – support transcription and translation as a part of the *L. monocytogenes* cold stress response (Bayles *et al.*, 1996; Schmid *et al.*, 2009).

Alkali and acid stress cause damage to bacterial DNA, cell membranes, enzymes, and energy metabolism by altering cell ion influx and efflux (Cotter & Hill, 2003; Soni *et al.*, 2011; Smith *et al.*, 2013). Acids can naturally occur in foodstuffs or can be used as preservatives, while several acidic and alkaline compounds are used in sanitizers and disinfectants of the food-processing environments. As *L. monocytogenes* is capable of growing over the broad pH range of 4.3–9.6 (Gray & Killinger, 1966), growth can only be inhibited by pH in highly acidic foods or with appropriate combinations of inhibitory concentration and duration of action for sanitizers in food-related environments. Restoration of pH homeostasis via membrane transport functions is an essential part of acid and alkali stress response of *L. monocytogenes* (Cotter & Hill, 2003; Soni *et al.*, 2011; Smith *et al.*, 2013).

Factors contributing to stress responses

Major groups of established stress response factors of *L. monocytogenes* are summarized in Table 1. Furthermore, several acclimation proteins of *L. monocytogenes* and other genetic loci with varying metabolic or yet unknown functions have been phenotypically linked to stress conditions relevant to the food chain (Soni *et al.*, 2011; Burgess *et al.*, 2016; NicAogáin & O'Byrne, 2016; Bucur *et al.*, 2018). Involvement of motility-related genes, for example, has been implicated in cold stress responses of *L. monocytogenes* (Mattila *et al.*, 2011; Markkula *et al.*, 2012a).

Many factors that mediate stress responses also play a role in the virulence of *L. monocytogenes* (Rouquette *et al.*, 1996; Wiedmann *et al.*, 1998; Cotter *et al.*, 1999; Kallipolitis & Ingmer, 2001; Kallipolitis *et al.*, 2003; Kazmierczak *et al.*, 2003; Chaturongakul *et al.*, 2008). For instance, ClpC and ClpP, belonging to heat shock class III proteins, are involved in intracellular growth and tolerance of *L. monocytogenes* towards high temperatures and salinity (Rouquette *et al.*, 1996; Gaillot *et al.*, 2000). Regulatory networks of *L. monocytogenes* consist of transcriptional regulators, such as CtsR, HrcA, and σ-factors, that activate and repress cellular processes, including stress response and virulence (Nair *et al.*, 2000; Chaturongakul & Boor, 2004; Hu *et al.*, 2007a; Hu *et al.*, 2007b; Toledo-Arana *et al.*, 2009; Chaturongakul *et al.*, 2011; Mattila *et al.*, 2012; Guariglia-Oropeza *et al.*, 2014; Liu *et al.*, 2019). The regulation of stress responses in *L. monocytogenes* also involves two-component systems that sense and mediate responses to external conditions

via signal transduction (Cotter *et al.*, 1999; Kallipolitis & Ingmer, 2001; Kallipolitis *et al.*, 2003; Pöntinen *et al.*, 2015; Pöntinen *et al.*, 2017).

The general stress response-related genes of *L. monocytogenes* reside in the chromosome. A third of *L. monocytogenes* strains carry plasmids (Lebrun *et al.*, 1992; McLauchlin *et al.*, 1997; Hingston *et al.*, 2017), where genes involved in antibiotic, benzalkonium chloride, and heavy metal resistance have been described (Poyart-Salmeron *et al.*, 1990; Lebrun *et al.*, 1992; Hadorn *et al.*, 1993; Lebrun *et al.*, 1994; Elhanafi *et al.*, 2010; Jiang *et al.*, 2016; Kremer *et al.*, 2017). *L. monocytogenes* plasmids are also reported to carry genes associated with oxidative stress response (Kuenne *et al.*, 2010; Liang *et al.*, 2016). Carriage of resistance genes by these self-replicating mobile genetic elements is a double-edged sword; while replication and trasmission of plasmids require resources from bacterial cells, they may also provide useful accessory acclimation mechanisms (Frost *et al.*, 2005; Rankin *et al.*, 2010). The presence of plasmids, which is common among food-related and environmental *L. monocytogenes* isolates (Lebrun *et al.*, 1992; McLauchlin *et al.*, 1997), has been associated with acid tolerance of *L. monocytogenes* and its sensitivity to cold and salt (Hingston *et al.*, 2017). Nonetheless, accessory mechanisms rendering some *L. monocytogenes* strains more resistant than others to stressors encountered in the food chain have seldom been reported (Hingston *et al.*, 2019a).

Stress adaptation and cross-adaptation

Exposure to sublethal levels of particular stresses has been shown to increase the tolerance of *L. monocytogenes* towards subsequent similar or lethal levels of stress, and, in some cases, evoke cross-adaptation to another stress condition (Farber & Brown, 1990; Lou & Yousef, 1997; Phan-Thanh *et al.*, 2000; Hill *et al.*, 2002; Faleiro *et al.*, 2003; Lundén *et al.*, 2003a; Skandamis *et al.*, 2008). Mild heat stress can cause adaptation of *L. monocytogenes* towards subsequent heat stress and cross-adaptation to other stressors encountered in food production, such as NaCl (Farber & Brown, 1990; Lou & Yousef, 1997; Lin & Chou, 2004; Sergelidis & Abrahim, 2009). Vice versa, following osmotic or cold stress, increased heat tolerance can ensue (Doyle *et al.*, 2001; Skandamis *et al.*, 2008; Hingston *et al.*, 2019b). Low pH and temperature have been linked to strain-specific salt tolerance (Faleiro *et al.*, 2003; Van Der Veen *et al.*, 2008), and the same stress tolerance mechanisms have indeed been reported in salt, acid, and cold conditions (Schmid *et al.*, 2009; Soni *et al.*, 2011). Adaptation to NaCl osmotic stress can also increase desiccation survival (Truelstrup Hansen & Vogel, 2011). Exposure to sublethal levels of disinfecting agents can lead to prolonged adaptation and cross-adaptation of *L. monocytogenes* towards disinfectants (Lundén *et al.*, 2003a). The adaptive and cross-adaptive responses of *L. monocytogenes* may enhance the ability of the bacterium to overcome food-processing hurdles, i.e., subsequent bacterial control steps utilized during food production and storage (Hill *et al.*, 2002; Ferreira *et al.*, 2014).

Table 1. Major groups of *Listeria monocytogenes* stress response factors involved in temperature, osmotic, or pH stress.

Group	Examples of factors	Description	Reference
Two-component systems	LisRK, CesRK, YycFG, LiaSR, AgrCA, VirRS	Response regulator–histidine kinase sensory regulation systems	Cotter *et al.* 1999; Kallipolitis & Ingmer 2001; Kallipolitis *et al.* 2003; Chan *et al.* 2008; Pöntinen *et al.* 2015 & 2017
Alternative sigma factors	σB, σC, σH, σL	Regulators of several stress response genes	Wiedmann *et al.* 1998; Kazmierczak *et al.* 2003; Chaturongakul *et al.* 2011; Mattila *et al.* 2012; Liu *et al.* 2019
Other regulatory factors	CtsR	Repressor of class III heat shock genes	Nair *et al.* 2000; Hu *et al.* 2007b
	HrcA	Repressor of class I heat shock genes	Hu *et al.* 2007a
	RsbT, RsbV	Regulators of σB	Chaturongakul & Boor 2004; Soni *et al.* 2011
Cold shock domain proteins	CspA, CspB, CspD, CspL	Nucleic acid chaperones	Bayles *et al.* 1996; Schmid *et al.* 2009
Heat shock class I proteins	GroES, GroEL, DnaK	Chaperones, proteases	Hanawa *et al.* 2000; Hu *et al.* 2007a
Heat shock class III proteins	ClpB, ClpC, ClpE, ClpP, ClpY	ATP-dependent proteases, chaperones	Rouquette *et al.* 1996; Gaillot *et al.* 2000; Nair *et al.* 2000; van der Veen 2007; Hu *et al.* 2007b
Class II stress response proteins	Ctc, HtrA	General stress response proteins	Duché *et al.* 2002a & 2002b; van der Veen *et al.* 2007; Wonderling *et al.* 2004
Osmolyte transporters	OpuC, Gbu, BetL	L-carnitine and glycine betaine transporters	Fraser *et al.* 2000; Duché *et al.* 2002a & 2002b; Angelidis & Smith 2003a & 2003b
DEAD-box RNA-helicases	lmo0866, lmo1722, lmo1450	RNA metabolism	Chan & Wiedmann 2009; Markkula *et al.* 2012a & 2012b

Biofilms, persistence, and stress tolerance

Biofilm-forming bacteria can adhere to each other and surfaces while producing extracellular polymeric substances to protect them from external stressors (Davey & O'Toole, 2000). In biofilms, the sharing of nutrients, collaborative removal of metabolites, and horizontal gene transfer are facilitated, which also increases bacterial persistence in potentially hostile environments (Davey & O'Toole, 2000). Bacterial adherence and formation of biofilms on food contact surfaces can compromise food quality and safety.

As *L. monocytogenes* readily adheres to surfaces (Spurlock & Zottola, 1991; Norwood & Gilmour, 1999; Lundén *et al.*, 2000; Lundén *et al.*, 2002; Chae *et al.*, 2006), contamination of the food-processing environment poses a considerable threat to product contamination (Autio *et al.*, 1999; Norton *et al.*, 2001a; Lundén *et al.*, 2002). Some *L. monocytogenes* strains have persisted in food-processing facilities for years (Miettinen *et al.*, 1999a; Norton *et al.*, 2001a; Hoffman *et al.*, 2003; Lundén *et al.*, 2003b; Keto-Timonen *et al.*, 2007; Malley *et al.*, 2013). *L. monocytogenes* has been reported to form biofilms in conditions and surfaces simulating food-processing environments (Chavant *et al.*, 2002; Piercey *et al.*, 2016; Papaioannou *et al.*, 2018) and is described to better tolerate different stressors and sanitizing agents within matured mono-species biofilms than as planktonic or newly adhered cells (Robbins *et al.*, 2005; Nilsson *et al.*, 2011; Truelstrup Hansen & Vogel, 2011; Hingston *et al.*, 2013; Piercey *et al.*, 2016) or in mixed-species biofilms (Daneshvar Alavi & Truelstrup Hansen, 2013; Papaioannou *et al.*, 2018).

The significance of mature biofilms in the ecology of *L. monocytogenes* in food-processing facilities has been a source of debate, as some theories emphasize microbial retention on surfaces at harborage sites as the cause of *L. monocytogenes* persistence (Carpentier & Cerf, 2011; Valderrama & Cutter, 2013; Ferreira *et al.*, 2014). In any case, the stress protection provided by biofilms may contribute to the presence of augmented quantities of *L. monocytogenes* cells on surfaces. This could increase the level of contamination transferred to foods, even if *L. monocytogenes* biofilm cells transferred less efficiently to foodstuffs than non-biofilm cells (Truelstrup Hansen & Vogel, 2011).

Strain variability of stress tolerance

L. monocytogenes isolates originating from different environments and hosts and exhibiting differing phenotypes, e.g., biofilm formation (Kadam *et al.*, 2013; Valderrama *et al.*, 2014), or genotypes, such as stress resistance genes (Moura *et al.*, 2016), often also differ in their genetic lineage, sublineage, and serotype (Trott *et al.*, 1993; Norton *et al.*, 2001b; Orsi *et al.*, 2011; Haase *et al.*, 2014; Maury *et al.*, 2016; Maury *et al.*, 2019). The identification of persistent strains, i.e., repeated isolation of identical *L. monocytogenes* strains from the same food-processing facilities (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Dauphin *et al.*, 2001; Norton *et al.*, 2001a; Vogel *et al.*, 2001b; Lundén *et al.*, 2002; Hoffman *et al.*, 2003; Lundén *et al.*, 2003b; Keto-

Timonen *et al.*, 2007; Malley *et al.*, 2013), and the occurrence of predominantly lineage II strains in food-associated environments (Hellström *et al.*, 2007; Orsi *et al.*, 2011; Lopez-Valladares *et al.*, 2018) have encouraged researchers to seek differences in stress tolerance and biofilm formation between persistent and sporadically occurring *L. monocytogenes* strains. Although higher acid tolerance and initial adherence to surfaces have been reported in persistent strains than in sporadic strains (Norwood & Gilmour, 1999; Lundén *et al.*, 2000; Lundén *et al.*, 2008), no specific "persistence phenotype" related to *L. monocytogenes* stress tolerance has yet been recognized (Carpentier & Cerf, 2011; Ringus *et al.*, 2012; Ferreira *et al.*, 2014).

Nevertheless, differences between *L. monocytogenes* strains have been described in their tolerance towards various stresses relevant to the food chain, including heat (Doyle *et al.*, 2001; Lin & Chou, 2004; Lianou *et al.*, 2006; Lundén *et al.*, 2008) and osmotic stress (Faleiro *et al.*, 2003; Van Der Veen *et al.*, 2008; Bergholz *et al.*, 2010; Ribeiro & Destro, 2014; Magalhães *et al.*, 2016; Hingston *et al.*, 2017). While tolerance of *L. monocytogenes* strains of lineage I towards salt and sensitivity of serotype 4b strains towards heat have been implied (Lianou *et al.*, 2006; Van Der Veen *et al.*, 2008; Bergholz *et al.*, 2010; Ribeiro & Destro, 2014), species-level conclusions on phenotypic diversity should be made cautiously if experiments rely on a small number of strains (Lianou *et al.*, 2006; Kadam *et al.*, 2013; Lianou & Koutsoumanis, 2013). In addition to understanding population diversity, strain variability of stress tolerance has implications on the selection of strains for challenge tests to estimate shelf lives of food products (Uyttendaele *et al.*, 2004; Lianou & Koutsoumanis, 2013). Extensive datasets are required to elucidate the intra-species variability of *L. monocytogenes* stress tolerance, including lineage-associated traits, which are currently not comprehensively understood.

2.2.2 Methods to investigate bacterial stress responses

Given that stress conditions either decelerate bacterial growth or affect the survival of bacterial cells, stress tolerance and resistance are measured by growth ability and survival under stress. Studying the underlying genetic mechanisms of stress responses requires the investigation and specification of a bacterial stress phenotype and linking it to genetic data.

Phenotypic studies on growth and survival

Survival from lethal stressors can be quantified by $\log_{10}$-reductions of bacterial viable counts (Ben Embarek & Huss, 1993; Lundén *et al.*, 2008; Skandamis *et al.*, 2008) and indirectly via minimum inhibitory concentrations (Firsov *et al.*, 1997; Aase *et al.*, 2000; Soumet *et al.*, 2005; Cebrián *et al.*, 2014; Ebner R. *et al.*, 2015). Studies reporting thermal death times also utilize D-values and z-values (Ben Embarek & Huss, 1993; Doyle *et al.*, 2001) that correspond, respectively, to the time in minutes required for a temperature to kill 90% ($\log_{10}$-cycle) of the population, and the temperature increase in degrees

required for a 10-fold (log_{10}) reduction of the D-value. Minimum and maximum growth temperatures also describe tolerance towards temperature stress (Junttila *et al.*, 1988; Hinderink *et al.*, 2009; Markkula *et al.*, 2012a).

Traditionally, the measurement of bacterial growth utilizes viable cell counts from cultures monitored over time, allowing for visualization of growth curves. A downward-sloping logarithmic inactivation curve follows exposure to lethal stress, while susceptibility to mild stress can be estimated by increases in lag phase, decreases in growth rate, and maximum growth level of the sigmoidal growth curve. When mathematically modeling growth under stress, tolerance can be quantified by growth parameters (Jason, 1983; Gibson *et al.*, 1987; Zwietering *et al.*, 1990; Baranyi & Roberts, 1994; Baranyi & Roberts, 1995; Buchanan *et al.*, 1997; Peleg & Corradini, 2011; Huang, 2013; Esser *et al.*, 2015). Common kinetic parameters comprise the following: lag time (lag phase, λ) representing the time period before the beginning of growth; maximum specific growth rate (growth rate, μ) derived from the slope of the logarithmic growth curve; asymptotic growth level (often abbreviated as "A") as the maximum level the population reaches by the stationary phase; and area under the curve (AUC) corresponding to the surface area below the growth curve (Korkeala *et al.*, 1992; Firsov *et al.*, 1997; Kahm *et al.*, 2010; Peleg & Corradini, 2011). As some models assume parameter correlations or do not fit certain types of data, the selection of a suitable parameter estimation approach for each study has been emphasized (Zwietering *et al.*, 1990; López *et al.*, 2004; Peleg & Corradini, 2011; Pla *et al.*, 2015).

The laborious nature of viable cell counts has led to the use of alternative technologies in growth experiments such as the widely utilized turbidity via absorbance (optical density, OD) measurements (Koch, 1970; Korkeala *et al.*, 1992; Sokolovic *et al.*, 1993; Augustin *et al.*, 1999; Faleiro *et al.*, 2003; Magalhães *et al.*, 2016; Hingston *et al.*, 2017; Keto-Timonen *et al.*, 2018). Optical density is measured by light transmitted through a sample, where the detection of turbidity depends upon equipment sensitivity and cell densities. Multiple scattering of light occurs at high cell densities, which increases the probability of light beams detected, and therefore, may underestimate turbidity (Koch, 1970; Stevenson *et al.*, 2016). Additionally, the delayed detection of bacterial growth at low cell densities by OD measuring equipment may lead to inaccurate lag time estimations (Dalgaard & Koutsoumanis, 2001). It is noteworthy that an apparent increase in cell size without an increase in cell numbers may increase the OD in high stress conditions (Stevenson *et al.*, 2016). *L. monocytogenes* cells under stress may elongate to form filaments that are divided by septa (Jørgensen *et al.*, 1995; Zaika & Fanelli, 2003; Hazeleger *et al.*, 2006) and consist of several normal-sized cells on the verge of division (Hazeleger *et al.*, 2006). Consequently, a potential increase in OD caused by these *L. monocytogenes* filaments corresponds to cell numbers.

Kinetic parameters calculated from OD measurements have been shown to systematically deviate from parameters obtained using viable counts (Dalgaard *et al.*, 1994). Thereby, comparison with traditional viable counts

and calibration of OD measurements have been performed (McClure *et al.*, 1993; Dalgaard *et al.*, 1994; Augustin *et al.*, 1999; Dalgaard & Koutsoumanis, 2001; Francois *et al.*, 2005; Pla *et al.*, 2015; Stevenson *et al.*, 2016). Conversely, if research focuses on the comparison of growth patterns, i.e., growth parameters of several strains relative to one another instead of their absolute values, similar results can be obtained by viable cell counts and OD measurements (Horáková *et al.*, 2004; Pla *et al.*, 2015).

As illustrated in this section, several methodological choices and considerations must be made to perform bacterial growth and stress tolerance experiments. Additionally, strain variability of growth ability should be differentiated from biological variability within individual strains and technical variability within experiments (Aryani *et al.*, 2015). However, execution of the entire data collection and analysis protocol and its repercussions on the comparability of bacterial growth experiments and reliability of stress tolerance studies have not been widely discussed in publications.

Identification of stress-related genetic mechanisms

Common approaches used in the identification of bacterial genetic mechanisms may roughly be categorized as follows: gene expression, genetic modification, and genomic analyses. Depending on the methodology, the level of evidence achieved for the genotype-phenotype interaction is association or causality, the latter of which can be accomplished by fulfilling the molecular Koch's postulates (Falkow, 2004). In the case of stress responses, these postulates could be modified to read: (i) the investigated stress phenotype should be associated with tolerant/resistant strains, and the genetic trait in question should be present in tolerant/resistant strains but absent in other strains of the bacterial species; (ii) inactivation/removal of the genetic trait should result in a quantifiable loss of stress tolerance/resistence; and (iii) introduction of the genetic trait should result in stress tolerance/resistence.

The expression of several *L. monocytogenes* genes has been associated with temperature, osmotic, and pH stress by transcriptomic and proteomic analyses (Sokolovic *et al.*, 1993; Bayles *et al.*, 1996; van der Veen *et al.*, 2007; Chan *et al.*, 2008; Schmid *et al.*, 2009; Mattila *et al.*, 2011; Soni *et al.*, 2011; Markkula *et al.*, 2012a; Pöntinen *et al.*, 2015). These analyses focus on gene expression by examining RNA transcribed or protein translated upon exposure to an investigated stressor. Conversely, methodologies of genetic modification, such as insertional mutagenesis (Camilli *et al.*, 1990), have associated specific or random inactivated genetic loci with an altered *L. monocytogenes* stress tolerance phenotype (Cotter *et al.*, 1999; Kallipolitis & Ingmer, 2001; Van Der Veen *et al.*, 2009; Hingston *et al.*, 2015). Additionally, deletion mutants combined with the complementation of the identified putatively stress-related genes have confirmed their causality in *L. monocytogenes* stress tolerance (Cotter *et al.*, 1999; Dussurget *et al.*, 2002;

Kazmierczak *et al.*, 2003; Schmid *et al.*, 2009; Markkula *et al.*, 2012b; Pöntinen *et al.*, 2017).

The development of DNA sequencing technologies and analysis methods has paved the way to genomic approaches for studying bacterial stress tolerance mechanisms. Genomic comparison of *L. monocytogenes* strains has yielded associations between particular phenotypes, MLST-types, and the presence or absence of *a priori* defined stress- and virulence-related genetic loci (Ebner R. *et al.*, 2015; Maury *et al.*, 2016; Hingston *et al.*, 2017). Conversely, genome-wide association studies (GWAS) provide ways to statistically associate also previously unknown variants derived from a large number of whole-genome sequences with binary or continuous bacterial phenotypes (Farhat *et al.*, 2013; Lees & Bentley, 2016; Lees *et al.*, 2016). In principle, a whole-genome sequence comparison of a closely related stress-resistant and stress-sensitive strain may identify variants present in one but absent in the other, hence indicating an association with the stress response. However, a large number of strains is required for statistical inference from genome-wide comparisons (Falush & Bowden, 2006; Read & Massey, 2014; Chen & Shapiro, 2015). Correspondingly, adopting emergent large-scale genome-wide approaches in the search for putative *L. monocytogenes* stress tolerance loci would first require the generation of reliable high-throughput data on the strain variability of *L. monocytogenes* stress phenotypes.

2.3 *L. monocytogenes* and the food chain

Stress tolerance characteristics of *L. monocytogenes* are advantageous in conditions encountered in the food chain and render traditional bacterial control measures, such as salting, refrigerating, and modified atmospheric packaging, insufficient against the bacterium. Stress tolerance and adherence to surfaces also facilitate the survival, colonization, and persistence of *L. monocytogenes* in food-processing environments. Consequently, *L. monocytogenes* has the reputation of being a problematic contaminant of food-processing facilities. RTE fish products are considered one of the characteristic vehicles of *L. monocytogenes*. Gravad and cold-smoked fish products are regarded as especially risky since they do not undergo listericidic heat treatments during processing or before consumption.

2.3.1 *L. monocytogenes* in food-processing facilities

Contaminated processing environments contribute to postprocessing contamination of food products, i.e., cross-contamination via the processing environment and machinery (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Dauphin *et al.*, 2001; Norton *et al.*, 2001a; Lundén *et al.*, 2002; Lundén *et al.*, 2003b; Keto-Timonen *et al.*, 2007; Bērziņš *et al.*, 2010). Food-processing facilities may suffer from persistent contamination characterized

by repeated isolation of the same *L. monocytogenes* strains from processing environments over a period of months or years (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Dauphin *et al.*, 2001; Norton *et al.*, 2001a; Vogel *et al.*, 2001b; Lundén *et al.*, 2002; Hoffman *et al.*, 2003; Lundén *et al.*, 2003b; Keto-Timonen *et al.*, 2007; Malley *et al.*, 2013). *L. monocytogenes* contamination of processing facilities can be difficult to eliminate without stringent sanitation measures (Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Lundén *et al.*, 2002; Lappi *et al.*, 2004b; Nakamura *et al.*, 2006; Bērziņš *et al.*, 2010). Notably, transfer of contaminated equipment can spread *L. monocytogenes* from one processing plant to another despite routine cleaning and disinfection of the equipment before use (Lundén *et al.*, 2002). On occasion, the control of *L. monocytogenes* in processing facilities may require structural renovations and the replacement of equipment or entire processing lines (Miettinen *et al.*, 1999a; Lundén *et al.*, 2002; Lappi *et al.*, 2004b; Keto-Timonen *et al.*, 2007). Sections 2.4 and 2.5 discussing *L. monocytogenes* in fish-processing plants elaborate on the contamination and control of the bacterium in fish production.

2.3.2 Vehicles of listeriosis outbreaks

The ecology of *L. monocytogenes* in the food chain is characterized by postprocessing contamination (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Dauphin *et al.*, 2001; Norton *et al.*, 2001a) and survival from mild heat treatments (Farber & Brown, 1990; Lou & Yousef, 1997; Sergelidis & Abrahim, 2009). Thereby, highly processed RTE products that do not undergo listericidal or successive heating before consumption, such as gravad and cold-smoked fish, cooked deli meats, and soft cheeses, are particularly risky for contracting listeriosis (Lopez-Valladares *et al.*, 2018; Ricci *et al.*, 2018). However, essentially any raw materials and foodstuffs of animal and plant origin can contain *L. monocytogenes* due to soil, water, fecal, and processing contamination. The largest listeriosis outbreak thus far, involving 1019 laboratory-confirmed cases, occurred in South Africa via Bologna sausage, i.e., "polony" (Allam *et al.*, 2018). Traditionally, several outbreaks have been attributed to products made of meat (McLauchlin *et al.*, 1991; Goulet *et al.*, 1998; Mead *et al.*, 2006), seafood (Ericsson *et al.*, 1997; Brett *et al.*, 1998; Miettinen *et al.*, 1999b), and raw milk (Linnan *et al.*, 1988; Goulet *et al.*, 1995; Danielsson-Tham *et al.*, 2004). Pasteurized milk (Fleming *et al.*, 1985) and products thereof, such as cheese (Koch *et al.*, 2010), butter (Lyytikäinen *et al.*, 2000), and ice cream (Pouillot *et al.*, 2016) have also been implicated as vehicles. During the recent decade outbreaks have increasingly included novel vehicles not thought to support the growth of *L. monocytogenes* or to pose a particular risk of listeriosis (Buchanan *et al.*, 2017; Desai *et al.*, 2019), including cantaloupe melon (Laksanalamai *et al.*, 2012), diced celery (Gaul *et al.*, 2012), and caramel apples (Angelo *et al.*, 2017).

Listeriosis outbreaks by gravad and cold-smoked fish products

RTE gravad and cold-smoked fish products have been implicated in published listeriosis outbreaks, all of which have concerned the Nordic countries (Table 2). Lopez-Valladares *et al.* (2018) also mention an unpublished Swedish outbreak of 27 cases by *L. monocytogenes* serotype 1/2a mediated by gravad and smoked fish products originating from one producer. In most cases, the *L. monocytogenes* outbreak strains have been linked to specific fish-processing plants (Ericsson *et al.*, 1997; Nakari *et al.*, 2014; Gillesberg Lassen *et al.*, 2016; Schjørring *et al.*, 2017; European Centre for Disease Prevention and Control & European Food Safety Authority, 2019). Concurrence of persistent *L. monocytogenes* contamination of the processing environment and deficiencies in self-checking, hygiene, maintenance, processing, and *L. monocytogenes* monitoring practices were reported in the implicated fish-processing plants in Finland (Nakari *et al.*, 2014). Isolation of the outbreak strains from different product batches of the same producer and from the processing environment, machinery, and equipment implies that persistent postprocessing contamination may have induced the outbreaks (Ericsson *et al.*, 1997; Tham *et al.*, 2000; Nakari *et al.*, 2014; Gillesberg Lassen *et al.*, 2016; Schjørring *et al.*, 2017; European Centre for Disease Prevention and Control & European Food Safety Authority, 2019).

Table 2. Outbreaks by *Listeria monocytogenes* (*Lm*) linked to the consumption of gravad or cold-smoked fish products

Implicated fish product	No. of cases (deaths)	*Lm* serotype (MLST type)	Type of listeriosis	Country	Reference
Vacuum-packed gravad rainbow trout	9 (1+1[a])	4b	Invasive: septicemia, meningitis, materno-fetal	Sweden	Ericsson *et al.* 1997; Tham *et al.* 2000
Vacuum-packed cold-smoked rainbow trout	5 (0)	1/2a	Non-invasive: gastroenteritis	Finland	Miettinen *et al.* 1999
Gravad and cold-smoked fish	17[b] (ND[c])	1/2a	Invasive: septicemia, meningitis, materno-fetal	Finland	Nakari *et al.* 2014
Cold-smoked salmon	10 (4+1[a])	1/2a (ST391)	Invasive: septicemia, meningitis, materno-fetal	Denmark	Gillesberg Lassen *et al.* 2016
Cold-smoked halibut and trout	10 (3)	4b (ST6)	Invasive: septicemia, meningitis	Denmark	Gillesberg Lassen *et al.* 2016
Cold-smoked salmon[d]	7 (1)	(ST8)	ND[c]	Denmark, France	Schjørring *et al.* 2017
Cold-smoked fish[e]	22 (5)	(ST1247)	Invasive	Denmark, Estonia, Finland, France, Sweden	ECDC & EFSA 2019

[a]Neonatal death/stillbirth; [b]Of a total of 71 listeriosis cases (22 deaths) reported in 2010, persistent fish-processing plant outbreak strains appeared in 17; [c]ND = not described; [d]Products originated from Poland; [e]Products originated from Estonia

2.4 *L. monocytogenes* in the fish industry

Fish product contamination typically occurs in the fish-processing plants, where persistent *L. monocytogenes* contamination can develop under favorable conditions (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Norton *et al.*, 2001a; Vogel *et al.*, 2001b; Hoffman *et al.*, 2003; Autio *et al.*, 2004; Wulff *et al.*, 2006; Malley *et al.*, 2013; Nakari *et al.*, 2014). This section summarizes factors that predispose gravad and cold-smoked fish products to *L. monocytogenes* contamination (Fig. 1).

2.4.1 Influence of product, production, and storage characteristics on *L. monocytogenes* contamination

Raw materials, product composition, processing methods, storage, and consumption habits for gravad and cold-smoked fish products predispose them to *L. monocytogenes* contamination and growth of the bacterium during shelf life. Investigations performed in Finland by public food authorities have found *L. monocytogenes* in 12–32% of the tested gravad and cold-smoked fish products (Åberg *et al.*, 2008; Niskanen *et al.*, 2010) and in 3.6% of the samples taken from facilities and food contact surfaces of fish-processing plants (Summa *et al.*, 2016). Across many countries, *L. monocytogenes* prevalences of 0–61% have been reported in cold-smoked fish products and 14–50% in gravad fish products (Lyhs *et al.*, 1998; Johansson *et al.*, 1999; Hellström *et al.*, 2007; Lambertz *et al.*, 2012; Løvdal, 2015; Kramarenko *et al.*, 2016; Koskar *et al.*, 2019). The prevalences of *L. monocytogenes* observed in environmental and working hygiene samples and in products at different stages of production have varied largely among fish-processing plants (Table 3), and thus, comparisons and conclusions should be drawn cautiously from prevalences obtained from different facilities and sampling schemes (Zoellner *et al.*, 2018).

Salmon (*Salmo salar*) and rainbow trout (*Onchorhyncus mykiss*) are widely used species for gravad and cold-smoked fish products. In samples consisting of head, gills, skin, flesh, or guts of fresh unprocessed salmon and rainbow trout, *L. monocytogenes* prevalences of 0–45% (Eklund *et al.*, 1995; Rørvik *et al.*, 1995; Vaz-Velho *et al.*, 1998; Dauphin *et al.*, 2001; Vogel *et al.*, 2001b; Hoffman *et al.*, 2003) and 1.1–15% (Jemmi & Keusch, 1994; Vaz-Velho *et al.*, 1998; Autio *et al.*, 1999; Markkula *et al.*, 2005; Miettinen & Wirtanen, 2005), respectively, have been reported. Hoffman *et al.* (2003) described significant differences in *L. monocytogenes* contamination of raw fish depending on the fish species, with Norwegian and U.S. West Coast salmon exhibiting the highest prevalences (21% and 30%, respectively).

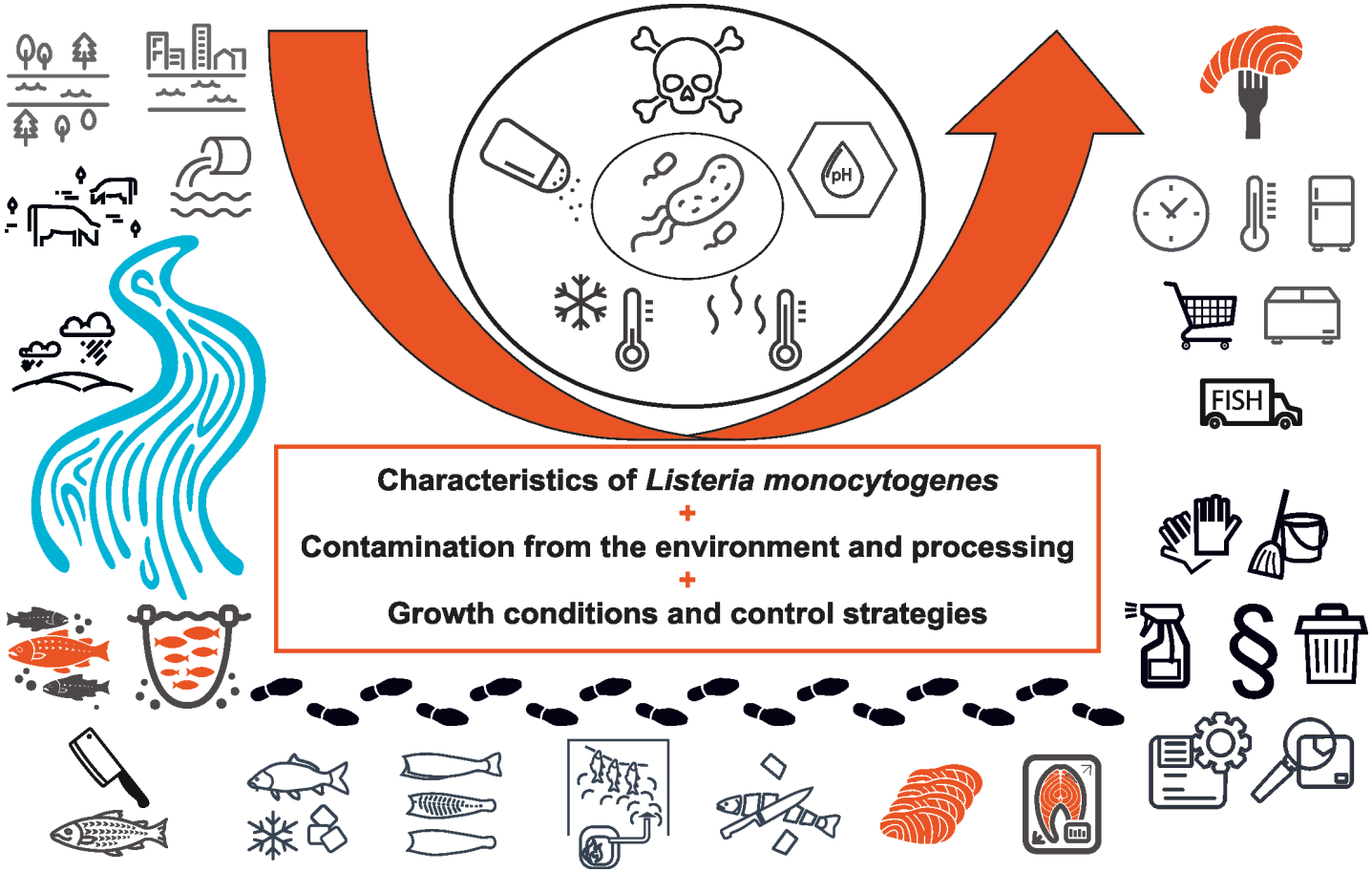

Figure 1. Summary of factors involved in the occurrence of *Listeria monocytogenes* in gravad and cold-smoked fish products.

Protein and fat contents vary by fish species, as does the predilection site of *L. moncytogenes* contamination. The higher fat content of salmon (10.6–17.2%) than cod (0.5–0.8%) has been hypothesized to protect *L. monocytogenes* from the effects of heating (Ben Embarek & Huss, 1993). In fresh rainbow trout, *L. monocytogenes* is described to be mainly located in the gills (Miettinen & Wirtanen, 2005), whereas high prevalences of *L. monocytogenes* have been reported on raw salmon skin (Eklund *et al.*, 1995; Dauphin *et al.*, 2001; Hoffman *et al.*, 2003). In addition to the type of fish, the quality of raw fish may play a role in contamination levels. *L. monocytogenes* has been isolated from skin bruises, but not from adjacent tissue of salmon arriving to processing facilities (Eklund *et al.*, 1995).

Close-to-neutral pH, high protein (nitrogen) content, and autolytic spoilage compounds make fish flesh a rich growth medium for bacteria, although the scarcity of glucose inhibits the growth of many. The microbes on fish originate from its water environments and may contain pathogens, such as *L. monocytogenes*, alongside native spoilage bacteria. Typical spoilage organisms of processed, vacuum-packaged fish products include psychrotrophic lactic acid bacteria, *Enterobacteriaceae*, and *Photobacterium phosphoreum* (Hansen *et al.*, 1995; Truelstrup Hansen & Huss, 1998; Franzetti *et al.*, 2003; Gimenez & Dalgaard, 2004; Jääskeläinen *et al.*, 2019). The presence of spoilage organisms mainly consisting of lactic acid bacteria can reduce the growth of *L. monocytogenes* from 10^8 to 10^2–10^4 cfu/g on cold-smoked salmon (Gimenez & Dalgaard, 2004). This growth reduction likely results from the growth of native lactic acid bacteria; according to a phenomenon called the Jameson effect, growth inhibition ensues as the total microbial population reaches its maximum population density in the food matrix (Ross *et al.*, 2000; Gimenez & Dalgaard, 2004; Hwang & Sheen, 2009; Daneshvar Alavi & Truelstrup Hansen, 2013). Consequently, favoring processing parameters that select for lactic acid bacteria may help inhibit the growth of *L. monocytogenes* in cold-smoked salmon (Tomé, 2007).

Processes involved in the production of gravad and cold-smoked fish products may include a combination of the following: freezing, thawing (melting), evisceration, head removal, filleting, salting (brining/curing), drying, cold-smoking, cooling, skinning, slicing, packaging, distribution, and cold storage (Tocmo *et al.*, 2014; Løvdal, 2015). None of the processes eliminate *L. monocytogenes* but aim at obtaining and maintaining sensory qualities and mitigating risks that can be managed. For instance, freezing of raw materials controls parasites and extends the time between slaughter and processing. Products can also be frozen and thawed, which may enhance the growth of *L. monocytogenes* as compared with non-freeze-thawed cold-smoked products (Kang *et al.*, 2012).

Dry salting, immersion brining, or injection brining are used for flavor enhancement and mild preservation of gravad and cold-smoked fish. Dry salting is performed by applying crude salt and possibly other spices, such as sugar and dill for gravad fish, on top of the fish fillets. Brining consists of

Table 3. Prevalence of *Listeria monocytogenes* in fish-processing establishments, their raw material, and ready-to-eat products.

Establishments	Time span of study	Prevalence (%) in environmental and working hygiene samples (no. of samples)	Prevalence (%) in raw fish (no. of samples)	Prevalence (%) in intermediate products (no. of samples)	Prevalence (%) in final products (no. of samples)	Reference
15 seafood-processing plants	3 years	1.6 (1349)	ND	ND	1.8 (272)	Leong *et al.* 2017
4 slaughterhouses	1 autumn	8.8 (57)	0 (34)	ND	ND	Løvdal *et al.* 2017
5 smokehouses, 3 other seafood facilities	1.5 years	2.5 (433)	ND	ND	3.8 (75)	Leong *et al.* 2015
2 smokehouses	1 year	21 (662)	19 (72)	ND	ND	Malley *et al.* 2013
1 smokehouse	6 years	17 (95)	24 (21)	14 (21)	12 (33)	DiCiccio *et al.* 2012
2 smokehouses	1 year	6.0 (165)	ND	13 (105)	5.6 (72)	Dimitrijevic *et al.* 2011
1 smokehouse	1 year	25 (444)[a]	ND	ND	ND	Chitlapilly Dass *et al.* 2010
1 smokehouse	5 days	1.0 (482)	ND	ND	0.3 (300)	Hu *et al.* 2006
1 smokehouse	3 years	22 (176)	0 (47)	ND	6.8 (59)	Nakamura *et al.* 2006
4 slaughterhouses, 4 smokehouses	3 months/1.5 years	26 (843)	25 (36)	ND	18 (74)	Wulff *et al.* 2006
4 smokehouses	3 years	12 (435)	16 (86)	ND	ND	Gudmundsdottir *et al.* 2005
1 smokehouse	4 days	47 (30)	ND	ND	ND	Eklund *et al.* 2004
4 smokehouses	2 years	23 (1144)	19 (518)	ND	7.9 (519)	Lappi *et al.* 2004

Table 3. *Continued.*

Establishments	Time span of study	Prevalence (%) in environmental and working hygiene samples (no. of samples)	Prevalence (%) in raw fish (no. of samples)	Prevalence (%) in intermediate products (no. of samples)	Prevalence (%) in final products (no. of samples)	Reference
4 smokehouses	1 year	13 (553)	3.8 (234)	ND	1.3 (233)	Thimothe *et al.* 2004
2 smokehouses	4 months	22 (512)	15 (315)	ND	ND	Hoffman *et al.* 2003
3 smokehouses	4 months	17 (64)	45 (33)	50 (22)	41 (32)	Dauphin *et al.* 2001
23 fish-processing plants	3 years	1.1 (943)	11 (18)	ND	14 (35)	Miettinen *et al.* 2001
3 smokehouses	6 months	21 (206)	8.8 (102)	17 (127)	12 (96)	Norton *et al.* 2001a
2 smokehouses	4 years	16 (1586)	1.6 (59)	ND	30 (228)	Vogel *et al.* 2001b
1 smokehouse	ND	20 (207)	1.1 (95)	55 (40)	100 (22)	Autio *et al.* 1999
1 smokehouse	1.5 years	15 (163)	0 (55)	ND	22 (37)	Johansson *et al.* 1999
7 smokehouses[b]	ND	23 (99)	ND	ND	79 (61)	Eklund *et al.* 1995
1 slaugherhouse, 1 smokehouse	8 months	21 (230)	5.4 (74)	26 (47)	11 (65)	Rørvik *et al.* 1995
3 fish farms incl. smokehouses	6 months	15 (262)	4.1 (72)	7.4 (108)	2.0 (49)	Jemmi & Keusch 1994

ND = No data available; [a]Includes products; [b]Environmental samples from 1 and product samples from 6 smokehouses.

immersing or injecting the fillets with a NaCl solution, i.e., brine. The salting step typically takes several hours or days and is performed in a chilled room. Higher pH and a_w and lower fat, water-phase salt, and phenolic content have been reported in brined than dry-salted cold-smoked salmon (Kang *et al.*, 2012). The NaCl content of the brine is usually 5–10%, resulting in a NaCl concentration of 1–4% in the products. Such salt concentrations do not inactivate *L. monocytogenes* (Van Der Veen *et al.*, 2008; Bergholz *et al.*, 2012; Ribeiro & Destro, 2014). Nonetheless, salt may reduce the survival and growth of *L. monocytogenes* in the prescence of smoke and phenolic compounds (Hwang, 2007; Porsby *et al.*, 2008).

The lack of a bactericidal thermal processing step is characteristic for the production of gravad and cold-smoked fish products. The survival of *L. monocytogenes* during heat treatments, including smoking, depends on the time-temperature combination. Hot smoking at above 45°C usually inhibits growth and may inactivate *L. monocytogenes* at temperatures of 58–70°C, particularly during long exposure times (Jemmi & Keusch, 1992; Ben Embarek & Huss, 1993). Although the process lasts for several hours or days, cold smoking temperatures (15–30°C) are not sufficient to inhibit the growth of *L. monocytogenes* – on the contrary, they are nearly its optimal temperatures. However, smoke has antimicrobial qualities that may somewhat deter *L. monocytogenes* growth (Hwang, 2007; Porsby *et al.*, 2008), but only when *L. monocytogenes* contamination resides on the fish skin and not in the flesh (Eklund *et al.*, 1995). Pellicle, a film-like surface forming on cold-smoked fish due to drying, may shield bacteria from the effects of smoke (Eklund *et al.*, 2004). The commonly used concentrations of dry or liquid smoke cannot be considered to eliminate *L. monocytogenes* (Eklund *et al.*, 1995; Hwang, 2007; Porsby *et al.*, 2008).

Modified atmosphere packaging reduces the level of oxygen to decrease undesirable bacterial growth in foodstuffs. Vacuum packaging removes the airspace around the product almost entirely, which selects for anaerobic bacteria. While many other microbes are inhibited by anaerobic atmospheres and refrigerating temperatures, these conditions do not impede *L. monocytogenes*. Although growth at refrigeration temperatures is slow, *L. monocytogenes* can grow from moderate to significant numbers during the typical 2- to 4-week cold storage of vacuum-packaged gravad and cold-smoked fish products (Rørvik *et al.*, 1991; Ericsson *et al.*, 1997; Johansson *et al.*, 1999; Uyttendaele *et al.*, 2009; Leong *et al.*, 2015; Kramarenko *et al.*, 2016), particularly since retail and consumer refrigerators are often kept at above 3°C (James *et al.*, 2008; Lundén *et al.*, 2014). Growth during consumer storage has been suggested to account for one-third of the European invasive listeriosis cases (Ricci *et al.*, 2018). Long storage times at households, high initial contamination level, relatively high refrigerator temperatures, and the presence of especially psychrotrophic *L. monocytogenes* strains, but less cold-tolerant spoilage microbes have been listed as major risk factors for cold-smoked salmon contamination with *L. monocytogenes* (Pouillot *et al.*, 2007).

Notably, initial contamination, handling of products, and stress tolerance qualities of contaminant strains influence the growth potential of *L. monocytogenes* throughout the processing and storage of gravad and cold-smoked fish products.

2.4.2 *L. monocytogenes* contamination patterns in the fish industry

The ecology of *L. monocytogenes* in the fish industry has been investigated by contamination analyses, the earliest of which included detection of *L. monocytogenes* from several samples taken from the processing environment and products (Jemmi & Keusch, 1994; Eklund *et al.*, 1995). Since the late 1990s, the development of molecular typing methods has allowed for the tracing and comparison of *L. monocytogenes* isolate genotypes throughout the fish-processing operations (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Norton *et al.*, 2001a; Vogel *et al.*, 2001b). The current theoretical background of *L. monocytogenes* contamination patterns in the fish industry can be recapitulated as follows: *L. monocytogenes* occasionally inevitably comes with raw materials into the fish-processing facilities (Eklund *et al.*, 1995; Vogel *et al.*, 2001a; Gudmundsdottir *et al.*, 2005; Markkula *et al.*, 2005), which then serve as the major site of product contamination (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Dauphin *et al.*, 2001; Norton *et al.*, 2001b; Vogel *et al.*, 2001b; Lappi *et al.*, 2004b; Thimothe *et al.*, 2004; Nakamura *et al.*, 2006; Chitlapilly Dass *et al.*, 2010; Dimitrijevic *et al.*, 2011; Di Ciccio *et al.*, 2012).

Contamination on fish farms

Given its occurrence in soils and watersheds, *L. monocytogenes* appears on fish farms, for instance, via brook and river waters (Miettinen & Wirtanen, 2006). Subsequently, contaminated water brings *L. monocytogenes* in contact with fish skin and gills, where the bacterium has predominantly been isolated (Dauphin *et al.*, 2001; Hoffman *et al.*, 2003; Miettinen & Wirtanen, 2005). Unlike cattle, fish do not seem to amplify *L. monocytogenes* contamination by colonization and fecal spread (Miettinen & Wirtanen, 2006). However, surface-contaminated raw fish can transport *L. monocytogenes* into the fish-processing chain (Eklund *et al.*, 1995; Markkula *et al.*, 2005). Raw materials originating from a fish farm that used earth ponds and river water from agricultural lands were suspected to contribute to the higher prevalence of *L. monocytogenes* observed in a fish-processing plant than in other similar facilities (Jemmi & Keusch, 1994).

Besides water sources, rainy weather may contribute to *L. monocytogenes* contamination of fish farms (Miettinen & Wirtanen, 2005; Miettinen & Wirtanen, 2006) – likely by runoff of contaminated surface water (Steele & Odumeru, 2004). *L. monocytogenes* contamination has been indicated to decrease rapidly in natural waters, but over several months in live fish (Hansen *et al.*, 2006; Miettinen & Wirtanen, 2006). Although *L. monocytogenes* contamination has been shown to vary between different fish

farms (Vaz-Velho *et al.*, 1998; Miettinen & Wirtanen, 2005; Hansen *et al.*, 2006), it also fluctuates notably within the same farm over time; the farm itself seems not to spread *L. monocytogenes*, as different strains appear in the farm environment and raw fish (Miettinen & Wirtanen, 2006). Given the dynamic nature of fish farm contamination, raw materials from all fish suppliers can be assumed to sometimes contain *L. monocytogenes*. However, operational and hygiene practices, such as poor cleaning of facilities, have been suggested to play a role in *L. monocytogenes* occurrence on fish farms (Jemmi & Keusch, 1994).

Contamination in fish-processing plants

Raw fish can contaminate products directly or indirectly via processing surfaces and handling; the latter, postprocessing contamination, predominates as a source of *L. monocytogenes* in fish products (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Norton *et al.*, 2001a; Nakamura *et al.*, 2004; Lappi *et al.*, 2004b; Thimothe *et al.*, 2004; Malley *et al.*, 2013; Leong *et al.*, 2017). Due to the potential presence of *L. monocytogenes* on the gut, gills, and skin of fish, evisceration, head removal, filleting, and skinning of raw fish may spread the contamination to fish flesh, processing equipment, and utensils. Correspondingly, contamination of raw fish by *L. monocytogenes* has been reported to appear after gutting at slaughterhouses (Løvdal *et al.*, 2017). Gravad and cold-smoked fish products undergo a high degree of processing, which predisposes them to postprocessing contamination (Fig. 2).

The complex fish-processing plant infrastructure provides enclosed niches that accumulate food debris and can become harborage sites, where *L. monocytogenes* forms persistent contamination while shielded from the surroundings and cleaning efforts (Autio *et al.*, 1999; Tompkin, 2002; Malley

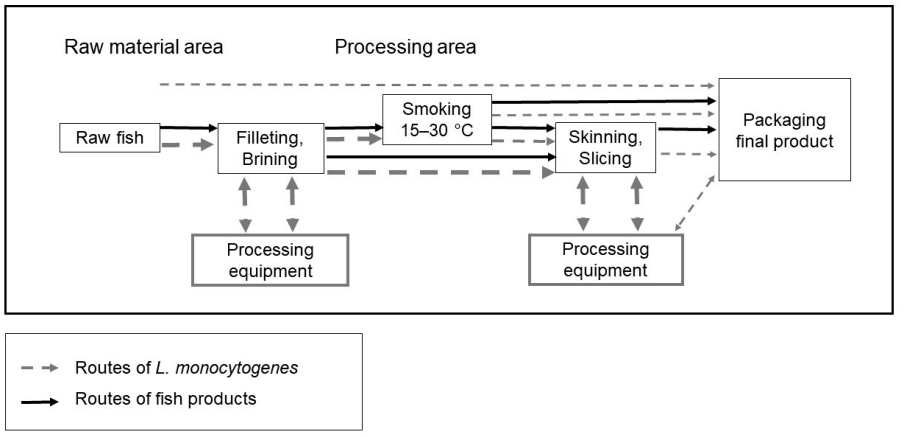

Figure 2 *Listeria monocytogenes* contamination routes in gravad and cold-smoked fish processing plants. Modified from Autio *et al.* (2004).

et al., 2015). The mobilization of debris and bacteria from niches during processing can bring *L. monocytogenes* in contact with products and spread contamination via transfer points such as gloves and other transiently contaminated surfaces (Malley *et al.*, 2015). Sites where *L. monocytogenes* has been found in contamination analyses of fish-processing plants are summarized in Fig. 3.

Poor hygienic design of, i.a., packaging and slicing machines, conveyors, dispensers, and coolers has been identified by food industry operators (Aarnisalo *et al.*, 2006). Complex processing machinery contains difficult-to-clean structures, such as blades, needles, and transporters, which easily become niches and harbor *L. monocytogenes* contamination (Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Miettinen *et al.*, 2001; Vogel *et al.*, 2001b; Lundén *et al.*, 2002; Lundén *et al.*, 2003b; Thimothe *et al.*, 2004; Gudmundsdottir *et al.*, 2005; Keto-Timonen *et al.*, 2007; Bērziņš *et al.*, 2010). Slicing and dicing machines (Autio *et al.*, 1999; Lundén *et al.*, 2002; Nakamura *et al.*, 2006; Aarnisalo *et al.*, 2007b; Malley *et al.*, 2013) and recirculated or injected brine (Autio *et al.*, 1999; Bērziņš *et al.*, 2010) are classical examples of niches and harborage sites. Injection brining can pass *L. monocytogenes* contamination into fish flesh, while after immersion brining, only fish skin has been found to be contaminated (Eklund *et al.*, 1995).

The repeated isolation of the same *L. monocytogenes* strains is characteristic for persistent *L. monocytogenes* contamination. Persistence of *L. monocytogenes* from 2.5 months to 4 years has been described in fish-processing facilities (Rørvik *et al.*, 1995; Dauphin *et al.*, 2001; Norton *et al.*, 2001a; Vogel *et al.*, 2001b; Hoffman *et al.*, 2003; Malley *et al.*, 2013; Nakari *et al.*, 2014). While sporadic *L. monocytogenes* strains also occur, persistent strains appear to predominate in fish-processing facilities (Malley *et al.*, 2013) – sometimes even the same strain in different processing plants (Wulff *et al.*, 2006). Depending on the fish-processing plant, filleting, brining, slicing, and packaging areas have been heavily contaminated (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Nakamura *et al.*, 2006; Dimitrijevic *et al.*, 2011; Di Ciccio *et al.*, 2012).

In addition to hazards related to facilities, machinery, and other equipment, working hygiene of the fish-processing plant personnel is a major factor in *L. monocytogenes* contamination. Fish raw material, employees' gloved hands, and contact surfaces both of fish and hands have been considered to play a role in the cycle of *L. monocytogenes* cross-contamination (Ivanek *et al.*, 2004). Job rotation, creating a pathway for *L. monocytogenes* to transfer from one department to another via movement of employees, has been identified as a strong risk factor for the occurrence of *L. monocytogenes* in smoked salmon (Rørvik *et al.*, 1997). Similarly, the lack of compartmentalization of the processing line has been indicated to be a risk factor in meat and poultry production (Lundén *et al.*, 2003b).

Aerial spread of *L. monocytogenes* in fish-processing plants has not been established (Autio *et al.*, 1999), but water sprays and splashes may spread

Equipment (16)	**Surfaces (18)**	**Machinery (16)**	**Utensils (7)**
Washing tubs/areas (7), e.g.,	Tables/table undersides (10), e.g.,	Slicing machine (12)	Knives (3)
Sink	*Filleting table*	Skinning machine (6)	Hand utensils (3), e.g.,
Curing fish wash	*Trim table*	Brining machine + needles (3)	*Filleting utensil*
Raw fish wash	*Sorting table*	Filleting machine (2)	Cutting boards (2), e.g.,
Rinsing tank	*Packaging table*	Head removal machine (2)	*Filleting board*
Water in fish crate	Door handles (3)	Meat-bone separating machine (2)	Deboning pin (1)
Water in washing tub	Condensate line and water (2)	Trimming machine (2)	
Crates (4)	Cutting table (2)	Packaging machine (2)	
Scale (3)	Non-contact surfaces (2)	Sorting machine (1)	**Personnel (10)**
Waste container (3)	Salting area and surfaces (2)	Portion machine (1)	Gloves (5)
Wheels of rolling carts (2)	Evisceration area (1)	Deboning machine (1)	Aprons (4)
Shelves (2), e.g.,	Filleting area (1)	Gutting machine (1)	Hands (4)
Drying shelf	Slicing surfaces (1)		Food handlers (2)
Trays (2), e.g.,	Packaging surfaces (1)		
Smoking tray	Waste disposal area (1)	**Transporters (9)**	**Brine (6)**
Forklift (2)	Hatch (1)	Conveyor belt (9), e.g.,	Recirculated brine (3)
Thawing tank and water (1)	Switches (1)	*Slicer conveyor belt*	Brine (3)
Sprinkler near head cutter (1)	Overhead waterline (1)	*Washing conveyor belt*	
Water douche (1)	Processing environment (1)	Transporters (1)	
Equipment (1)			
Ice packs (1)			
Mixer (1)	**Floors (10)**	**Drains (10)**	**Other (1)**
Smoking rack (1)	Floor mat (2)		Air (1)
Plastic pallet (1)	Pooled water (1)		Outdoor grounds (1)
Footstool (1)			

Figure 3 Sites of *Listeria monocytogenes* contamination in fish-processing facilities. Data in parentheses refer to the number of publications referenced in Table 3 that mention the isolation site in question.

Listeria spp. (Berrang & Frank, 2012), for instance, during cleaning. Running water on floors has been speculated to aggravate *L. monocytogenes* contamination (Hoffman *et al.*, 2003). Thawed waters from frozen raw fish may contain *L. monocytogenes* (Eklund *et al.*, 1995) and spread it, if handled unhygienically. Lack of sanitation, maintenance, and sampling of fish-processing plant facilities have also been reported in conjunction with *L. monocytogenes* contamination (Jemmi & Keusch, 1994; Miettinen *et al.*, 2001; Hoffman *et al.*, 2003; Nakari *et al.*, 2014). Thereby, while prior investigations have shed light on the contamination routes of *L. monocytogenes*, it is now important to study potential preventive measures – including operational practices and official food control procedures at fish-processing plants – and their association with the contamination of fish products by *L. monocytogenes*.

2.5 Prevention and control of *L. monocytogenes* in fish-processing plants

The control of *L. monocytogenes* in fish products encompasses the entire food chain: from minimizing initial contamination by hygienic handling at fish farms and factories to awareness and appropriate handling by consumers holding the fork (Luber *et al.*, 2011). Actions by fish-processing plants and their official control play a major role in preventing food safety non-compliances (violations), including *L. monocytogenes* contamination. On the one hand, food producers are responsible for taking actions to ensure food safety (EC no. 178/2002; Food Act 23/2006). On the other hand, compliance with food safety legislation is monitored, guided, and – if necessary – demanded and ordered by official food control authorities (EU no. 625/2017; EC no. 178/2002; EC no. 882/2004; Food Act 23/2006). Factors related to processing plant layout, machines, process parameters, working hygiene, sanitation, and overall compliance with food safety legislation and official food control are involved in *L. monocytogenes* contamination and food safety management (Rørvik *et al.*, 1997; Autio *et al.*, 1999; Miettinen *et al.*, 2001; Lundén *et al.*, 2003b; Lappi *et al.*, 2004b; Yapp & Fairman, 2006; Davies *et al.*, 2014; Läikkö-Roto & Nevas, 2014a). Consequently, rigorous interventions targeted at processing and working hygiene violations have been shown to reduce *L. monocytogenes* occurrence (Autio *et al.*, 1999; Lappi *et al.*, 2004b; Keto-Timonen *et al.*, 2007; Nakari *et al.*, 2014). Thereby, it can be assumed that an efficient self-checking system, i.e., the processing plant's own system for managing food safety (own-checks), and adequate official control measures are required for *L. monocytogenes* prevention. However, limited knowledge is available on the current execution of preventive measures, occurrence of non-compliances, and efficacy of official control in fish-processing plants.

2.5.1 Food safety legislation in Finland

Finland follows the food safety regulations of the European Commission (EC), which apply as such to all member states of the European Union (EU). In the legislative hierarchy, EC regulations precede national laws. The EC Directorate-General of Health and Food Safety is responsible for the EU legislation on food safety and monitors its implementation by auditing national food authorities. During the period investigated by this dissertation (2011–2015), the legal hygiene requirements concerning Finnish fish-processing plants and their official control included the EU legislation pertaining to food business operators and authorities, food hygiene, processing of foods of animal origin and their official control, and microbiological criteria for foodstuffs (EC no. 178/2002; EC no. 852/2004; EC no. 853/2004; EC no. 854/2004; EC no. 882/2004; EC no. 2073/2005).

The general principles of food law – including risk analysis, precaution, traceability, and transparency – and the responsibilities of food business operators on the safety of products and competent authorities on enforcement and monitoring – are laid down in EC no. 178/2002. At the time of this study, the general principles and requirements of official food control, including control systems, resources, documentation, and reporting, were given in EC no. 882/2004, according to which official control is to be transparent, equal, efficient, and effective, and carried out regularly, on a risk basis, with appropriate frequency and without warning. The regulations EC no. 852/2004, EC no. 853/2004, and EC no. 854/2004, referred to as the "hygiene package", issue the general and industry-specific hygiene requirements, good management practices, self-checking systems – which must lean on the principles of Hazard Analysis and Critical Control Points (HACCP) – and the official control of products of animal origin. The microbial criteria for food safety and process hygiene, including sampling and testing, are given in EC no. 2073/2005.

Some matters of food safety may only be issued by complementing national decrees. These include the specification of the competent authorities and their enforcement measures (Food Act 23/2006), notification and approval procedures of food establishments (Government decree on food control 420/2011), and additional self-checking system requirements for certain types of establishments, including fish-processing plants (Ministry of Agriculture and Forestry statute 795/2014). However, as the reformed official food control regulation entered the application phase in December 2019 (EU no. 625/2017), the complementing national legislation was amended and entered into force in January 2020. The EU regulation no. 625/2017 includes no major changes to the current preventive and control measures of *L. monocytogenes*. Nevertheless, the amendments to national legislation, including the omission of standalone written self-checking plans and the addition of novel control measures, may influence the control of *L. monocytogenes* in practice. Requiring a self-checking system but not a self-checking plan can lessen the administrative burden of food business operators but may also complicate the

performance and evaluation of systematic planning behind self-checking operations. In contrast, the scope of control measures available in official food control is broadened by the possibility to (i) obtain Compliance Reports from the Finnish Tax Administration to assess the reliability of food business operators and (ii) execute coercive measures based on such assessments of reliability. Poor compliance with statutory financial obligations has been found to correlate with non-compliance observed at food control inspections (Finnish Tax Administration, Grey Economy Information Unit, 2018). Therefore, the Compliance Report will give food control authorities additional tools to tackle non-compliances. Potential effects remain to be seen once the implementation and interpretation of the reformed legislation proceeds.

2.5.2 Official food control system in Finland

The area of food safety and control falls under the jurisdiction of the Finnish Ministry of Agriculture and Forestry. The implementation of the food safety legislation is planned, streered, developed, and undertaken by the Finnish Food Authority, which was known as the Finnish Food Safety Authority Evira until 31 December 2018. Under the guidance of this central authority, Regional State Administrative Agencies supervise and assess municipal food control units that are responsible for carrying out the official food control inspections of food establishments. The Finnish Food Authority draws up a national control plan for the official control of the food chain (Finnish Food Authority, 2010). This plan creates a framework for devising the municipal food control plans that specify the content of inspections, frequency and duration of control visits, sampling performed by the authorities, and evaluation of the realization of control plans.

The efficacy of official food control arises from regular risk-based inspections performed with frequencies and methods adequate to detect and remove non-compliances. Investigations of the costs, efficacy, and consistency of the Finnish food control system have identified differences among municipal food control units (Lepistö *et al.*, 2009; Tähkäpää *et al.*, 2009; Läikkö-Roto *et al.*, 2015; Heikkilä *et al.*, 2016; Läikkö-Roto *et al.*, 2016; Kettunen *et al.*, 2017b). The number of control objects per man-year has been found to vary from 148 to 628, and the number of objects inspected annually per man-year from 52 to 142 between food control units (Heikkilä *et al.*, 2016). Personnel resources have been deemed insufficient by 62% of unit heads (n = 16) and 48% of inspectors (n = 56) (Heikkilä *et al.*, 2016). Although Finnish food control units provide guidance material, inspection templates, and peer-support, the performance and efficacy of municipal food control is challenged by time constraints, lack of access to tacit knowledge, and inadequacy of management and workplace resources (Läikkö-Roto *et al.*, 2016). Management of personnel and food control revenues and presence of food expertise under the local environmental health care governance likely influence the way food control resources are perceived and play a role in

funding decisions and utilization of municipal resources (Tähkäpää *et al.*, 2008; Heikkilä *et al.*, 2016).

2.5.3 Guidelines for *L. monocytogenes* control in Finnish fish-processing plants

The Finnish Food Authority provides several non-binding national guidelines on official food control and interpretation of food safety legislation. Such guidelines pertaining to the official control of fish-processing plants include assessment of risk and inspection frequency (Finnish Food Authority, 2017a), risk-based official control of the food establishments and their self-checking systems (Finnish Food Authority, 2015a; Finnish Food Authority, 2015b), official control of fish products (Finnish Food Authority, 2017b), and microbiological criteria of foodstuffs (Finnish Food Authority, 2017c). A separate checklist has been in use to summarize the inspection of *L. monocytogenes* preventive measures in the self-checking systems of food business operators (Finnish Food Authority, 2012).

Since the research described in this dissertation was conducted, the Finnish food control inspection system has been reformed. The new system, 'Oiva', contains specific guidelines for conducting inspections, including a section for the evaluation of the *L. monocytogenes* self-checking system and sampling strategy (Finnish Food Authority, 2019b). In addition, official non-binding guidelines concerning *L. monocytogenes* are provided by international organizations (Codex Alimentarius Commission, 2007; Carpentier & Barre, 2012; European Comission, 2013; European Salmon Smokers Association, 2018). The Finnish food industry stakeholders have also issued non-official guidelines concerning self-checking systems and shelf lives in the fish industry (Finnish Food and Drink Industries' Federation, 2006; Finnish Food and Drink Industries' Federation, 2009).

2.5.4 *L. monocytogenes* control measures by fish-processing plants

The operations of Finnish fish-processing plants are organized and self-supervised by statutory self-checking systems, which comprise assembled plans, self-surveillance, and execution of prerequisite programs, hygienic protocols, and microbiological sampling. The tools for managing food safety risks outlined by the legislative framework include good hygiene and manufacturing practices, sanitation standard operating procedures, and hazard analysis and control principles based on HACCP. A functional self-checking system is dependent on the monitoring of operations, correction of observed violations, and verification of performed corrections by the fish-processing plants themselves.

The general principles of preventing *L. monocytogenes* contamination in food processing (Huss *et al.*, 2000; Luber *et al.*, 2011) apply to gravad and cold-smoked fish products (Table 4). Given the characteristics of *L.*

monocytogenes and production processes summarized in Sections 2.2 and 2.4, the emphasis of preventive measures in gravad and cold-smoked fish production is on hygiene, sanitation, sampling, and growth inhibition.

Growth inhibition

In the EU, the microbiological criteria concerning *L. monocytogenes* are stringent for food products that can support the growth of *L. monocytogenes* or are intended for risk populations (EC no. 2073/2005). Vacuum-packaged gravad and cold-smoked fish products are specified as RTE products able to sustain *L. monocytogenes* growth, and their shelf life is recommended to be kept at a maximum of 14 days under 3°C (Finnish Food Authority, 2017b). The producer must be able to demonstrate that the amount of *L. monocytogenes* in these products does not exceed 100 cfu/g during their shelf life, or otherwise *L. monocytogenes* must not be detected in products at processing (Finnish Food Authority, 2017c; EC no. 2073/2005). Conversely, the United States, among other countries, enforces a zero tolerance without exception for *L. monocytogenes* in RTE foods (Shank *et al.*, 1996; Jami *et al.*, 2014).

Alongside improving temperature control and shortening storage periods, re-formulating the composition of foodstuffs so that they no longer support the growth of *L. monocytogenes* has been assessed to reduce the risk of listeriosis (World Health Organization & Food and Agriculture Organization of the United Nations, 2004). As opportunity for temperature abuse may arise at retail or consumer refrigeration (Pouillot *et al.*, 2009; Luber *et al.*, 2011; Lambertz *et al.*, 2012; Lundén *et al.*, 2014; Dumitraşcu *et al.*, 2020), additional hurdles for growth prevention, such as biopreservation by selected lactic acid bacteria or bacteriophages and chemical preservation by nisin or salts of organic acids, have been considered with promising results (Nykänen *et al.*, 2000; Tomé *et al.*, 2007; Soni & Nannapaneni, 2010; Kang *et al.*, 2014; Tocmo *et al.*, 2014; Aymerich *et al.*, 2019). In the absence of a lethal heat treatment, exposure to other physical processes, such as irradiation, UV light, and high-

Table 4. Principles of *Listeria monocytogenes* prevention in food processing.

Aim	Preventive method
Avoidance of *L. monocytogenes* contamination, survival, and multiplication on processing surfaces and products	Design, layout, and maintenance of processing equipment and facilities; specific sanitation program
Reduction and inhibition of *L. monocytogenes* growth during shelf life	Hygiene in production, packaging, and storage processes
Monitoring of *L. monocytogenes* presence and sources; validation of sanitation program	Microbiological sampling of the processing environment and products

Compiled from Huss *et al.* (2000) and Luber *et al.* (2011).

pressure processing, has been discussed (Tocmo *et al.*, 2014; Bahrami *et al.*, 2020). Although efficient, irradiation may not be accepted by consumers, and irradiated fish products are currently not allowed on the market in Finland. Evidence on other physical methods in growth prevention of *L. monocytogenes* in RTE fish products is limited and somewhat inconsistent (Tocmo *et al.*, 2014; Løvdal, 2015; Bahrami *et al.*, 2020).

Concurrent implementation of both growth inhibition and contamination prevention is important for reducing the public health impact of listeriosis. Nevertheless, while potentially efficient, additional hurdles targeting shelf life should not inadvertently divert the focus of *L. monocytogenes* management away from contamination prevention towards mere growth control. Frequently reported infectious doses of *L. monocytogenes* range from 10^2 to 10^7 cfu/g (reviewed by Lopez-Valladares *et al.*, 2018). However, susceptibility to listeriosis depends on underlying health conditions (World Health Organization & Food and Agriculture Organization of the United Nations, 2004), and some foodstuffs implicated in listeriosis outbreaks involving immunocompromised individuals have contained <10 cfu/g of *L. monocytogenes* (Maijala *et al.*, 2001; Pouillot *et al.*, 2016). Given that product contamination typically originates from the processing environment (Rørvik *et al.*, 1995; Autio *et al.*, 1999), the presence of <100 cfu/g of *L. monocytogenes* in a food product could indeed be considered a processing failure that warrants interventions (Luber *et al.*, 2011). Consequently, environmental monitoring combined with hygiene and sanitation measures are required from a preventive perspective (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Vogel *et al.*, 2001b; Lundén *et al.*, 2002; Lappi *et al.*, 2004b; Nakamura *et al.*, 2006; Keto-Timonen *et al.*, 2007; Bērziņš *et al.*, 2010; Luber *et al.*, 2011; Malley *et al.*, 2015).

Environmental and product sampling

As fish-processing plants are often sources of *L. monocytogenes* contamination (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Dauphin *et al.*, 2001; Norton *et al.*, 2001b; Vogel *et al.*, 2001b; Lappi *et al.*, 2004b), sampling of the processing environment facilitates the orientation of control measures (Autio *et al.*, 1999; Lappi *et al.*, 2004b; Luber *et al.*, 2011; Malley *et al.*, 2013; Malley *et al.*, 2015). Contamination analyses use extensive sampling combined with molecular typing tools to match *L. monocytogenes* strains isolated from products to strains from processing facilities and machines, hence specifying the sources of product contamination in the processing environment (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Dauphin *et al.*, 2001; Norton *et al.*, 2001b; Vogel *et al.*, 2001b; Lundén *et al.*, 2003b; Lappi *et al.*, 2004b; Thimothe *et al.*, 2004; Nakamura *et al.*, 2006; Keto-Timonen *et al.*, 2007; Bērziņš *et al.*, 2010; Chitlapilly Dass *et al.*, 2010; Dimitrijevic *et al.*, 2011; Di Ciccio *et al.*, 2012; Malley *et al.*, 2013; Leong *et al.*, 2017). Correspondingly, a process called Seek & Destroy utilizes systematic aggressive environmental sampling to discover bacterial growth niches and *L.*

monocytogenes harborage sites (Butts, 2003; Malley *et al.*, 2015). In the process, facility and equipment areas at various levels of assembly are extensively sampled after normal sanitation for *L. monocytogenes* and indicator organisms. The sampling outcomes enable the definition of normal and periodic enhanced levels required from sanitation and disassembly, which can then be incorporated into the sanitation standard operating procedures. The Seek & Destroy process can be used for both eradication and prevention of *L. monocytogenes* (Butts, 2003; Malley *et al.*, 2015).

In addition to assessing harborage sites and sources of contamination, environmental sampling is used to monitor process hygiene (EC no. 2073/2005; Finnish Food Authority, 2012; Finnish Food Authority, 2017c; Finnish Food Authority, 2019b), for which sampling during production is commonly recommended by regulatory documents (Zoellner *et al.*, 2018). The Finnish and EU guidelines for *L. monocytogenes* self-checking procedures also recommend the monitoring of environmental samples mainly during or after processing and before sanitation (Carpentier & Barre, 2012; Finnish Food Authority, 2017a). This approach is considered to enhance the likelihood of detecting *L. monocytogenes* dislodged from harborage sites and to evade potential non-culturability of cells damaged by sanitizers (Carpentier & Barre, 2012), but it lacks the potential of thorough contamination analyses to discover and eliminate harborage sites and validate successful sanitation procedures. Thereby, the objective of sampling must be kept in mind when determining the number, timing, frequency, and sites of sampling for environmental monitoring schemes (Zoellner *et al.*, 2018).

A correlation between *L. monocytogenes* contamination in the environment and contaminated products of fish-processing plants has been reported (Lappi *et al.*, 2004b; Thimothe *et al.*, 2004). However, just as product samples alone cannot describe the functioning of a food safety management system (Zwietering *et al.*, 2016), environmental samples alone cannot confirm, whether the final products are indeed contaminated. The EU food safety limits focus on the presence and amount of *L. monocytogenes* in food products during their shelf life (EC no. 2073/2005). Finnish guidelines endorse augmenting product sampling, if *L. monocytogenes* is discovered in samples from the processing environment (Finnish Food Authority, 2017c). Non-compliance with the food safety limits for *L. monocytogenes* should lead to product recalls and the tracing of contamination sources.

Hygiene and sanitation

Poor maintenance and sanitation of processing equipment and facilities as well as unhygienic processing practices predispose to fish-processing environment and fish product contamination with *L. monocytogenes* (Rørvik *et al.*, 1997; Autio *et al.*, 1999; Miettinen *et al.*, 2001). Interventions targeted at hygiene, cleaning, and working practices of food industry operators, such as using appropriate sanitizers and disinfectants (Aarnisalo *et al.*, 2000; Aarnisalo *et al.*, 2007a), combining cleaning with hot water, air, and steam

(Autio *et al.*, 1999), and removing identified harborage sites, e.g., floor mats (Lappi *et al.*, 2004b) and cooler conveyors (Keto-Timonen *et al.*, 2007), have been successful in *L. monocytogenes* elimination and successive prevention. Based on the risk factors identified (see Section 2.4), avoidance of cross-contamination via water splashes and crossing pathways, enhancing hygienic working routines and sanitation, and improving hygienic design of the facilities, machinery, and equipment are consecutively considered to strengthen *L. monocytogenes* prevention (Rørvik *et al.*, 1997; Autio *et al.*, 1999; Vogel *et al.*, 2001b; Lundén *et al.*, 2002; Tompkin, 2002; Lundén *et al.*, 2003b; Nakamura *et al.*, 2004; Lappi *et al.*, 2004b; Thimothe *et al.*, 2004; Gudmundsdottir *et al.*, 2005; Keto-Timonen *et al.*, 2007; Nakari *et al.*, 2014).

Traditionally, the emphasis of *L. monocytogenes* preventive measures is on cleaning and disinfection protocols (Autio *et al.*, 1999; Huss *et al.*, 2000) which can be divided into (i) regular sanitation, (ii) sanitation of special targets, e.g., conveyor belts and machines for packaging, slicing, and salting, (iii) periodic thorough sanitation, and (iv) sanitation to eradicate established contamination (Finnish Food Authority, 2012). Dismantling of processing equipment and mechanical cleaning to remove debris are stressed as important regular cleaning practices. The sanitation of special targets can be facilitated by ultrasonic cleaning which is effective in reducing *L. monocytogenes* on, for instance, conveyor belts (Tolvanen *et al.*, 2007; Tolvanen *et al.*, 2009).

The occurrence of *L. monocytogenes* in environmental samples should lead to additional sampling and cleaning of the processing environment, including extensive dismantling of machinery and stringent sanitation measures (Autio *et al.*, 1999). While cleaning of equipment during production has been suggested to be related to reduced *L. monocytogenes* fish product contamination (Rørvik *et al.*, 1997), no reports specifically link in-between-process cleaning of machinery or periodic thorough sanitation practices to *L. monocytogenes* contamination. Overall, some associations between food industry operational practices and *L. monocytogenes* product contamination have been described, such as job rotation, injection brining, cold-smoking parameters, and application of specific control strategies (Rørvik *et al.*, 1997; Lappi *et al.*, 2004b; Bērziņš *et al.*, 2007). Additionally, high prevalence of contamination among fish-processors has elicited concern about how to evaluate the microbiological quality of their products (Rotariu *et al.*, 2014) However, comprehensive elucidation of current production practices and *L. monocytogenes* preventive measures in fish-processing plants requires further investigation.

Knowledge, behavior, and compliance

Fish industry operators have been reported to implement insufficient and non-compliant food safety practices (Lundén, 2013; Rotariu *et al.*, 2014). Targeted training aimed at understanding the underlying reasons for food safety actions may increase employee knowledge and commitment to *L. monocytogenes*

control (Hicks *et al.*, 2004). However, human behavior is not only affected by knowledge (Clayton *et al.*, 2002; Yapp & Fairman, 2006; Griffith, 2010). Everyday food safety actions are executed by food handlers, who have stated that lack of time, staff, and resources prevent the implementation of appropriate food safety practices (Clayton *et al.*, 2002). A recent systematic review concluded that factors related to food handlers' capabilities, motivation, opportunities, and sociodemographics compromise safe food handling; despite good knowledge and attitudes towards training, lapses in motivation and concentration in performing food safety practices occur (Thaivalappil *et al.*, 2018). Environmental factors, such as resources, policies, reminders, emotions, and social influences, affect the intentions and actions of food handlers (Thaivalappil *et al.*, 2018). The concept of food safety culture, i.e., positive or negative "workplace culture" towards food safety, illustrates how not only the individual food handler but the management that facilitates employee behavior is ultimately responsible for food safety performance (Griffith *et al.*, 2010; Griffith, 2010). Food safety culture has recently been associated with the predicted risk of *L. monocytogenes* contamination in retail deli environments (Wu *et al.*, 2020).

Underestimation of food safety risks appears to be common among food safety staff and management (Mortlock *et al.*, 1999; Clayton *et al.*, 2002; Thaivalappil *et al.*, 2018). In Finland, fish-processing plants have been reported to perceive the food safety risks of their operations as smaller than other food-processing plants (Nevas *et al.*, 2013), which contradicts the evident risk of *L. monocytogenes* posed by the processing of vacuum-packaged RTE fish products. Moreover, a decreased risk perception of food industry operators has been associated with a decreased likelihood of employing a HACCP-based food safety management system (Mortlock *et al.*, 1999) and an increased likelihood of non-compliance (Kaskela *et al.*, 2019). Therefore, more knowledge of present-day operational practices and occurrence of non-compliances in conjunction with *L. monocytogenes* fish product contamination is required to improve *L. monocytogenes* prevention in fish-processing plants.

2.5.5 *L. monocytogenes* control measures by official food control

L. monocytogenes sampling is the responsibility of food business operators, but municipal food control officials should also monitor *L. monocytogenes* contamination with official sampling. National and international surveillance of *L. monocytogenes* by authorities enables the linking of sporadic listeriosis cases scattered across time and geographical locations to potential sources and possible continuous outbreaks (Luber *et al.*, 2011; Nakari *et al.*, 2014; Gillesberg Lassen *et al.*, 2016; Schjørring *et al.*, 2017; European Centre for Disease Prevention and Control & European Food Safety Authority, 2019). This facilitates the recognition of problematic foodstuffs and food business operators and the allocation of risk-based food inspection resources.

Characterized as a highly risky operation, the processing of vacuum-packaged gravad and cold-smoked fish was considered to require 5–11 annual inspections in Finland in 2011–2014 (Finnish Food Authority, 2010), and currently, 3–12 inspections depending on the production quantity (Finnish Food Authority, 2017a).

As food control research is a relatively new field of science (Nevas & Korkeala, 2009), no detailed overview has been available on the efficacy of official food control in the fish industry. Nevertheless, inspections and interventions by authorities have succeeded at mitigating *L. monocytogenes* fish product contamination and outbreaks (Nakari *et al.*, 2014; Gillesberg Lassen *et al.*, 2016). Successful interventions by inspectors have included requests to send *L. monocytogenes* samples to an accredited laboratory, shorten gravad and cold-smoked fish product shelf lives to 14 days, strengthen hygienic procedures, replace equipment parts, and perform structural changes, renovations, and intensified cleaning, all of which have led to a decrease of *L. monocytogenes* from >100 cfu/g to <100 cfu/g in the product samples of two fish-processing plants (Nakari *et al.*, 2014). On another occasion, a marketing and production ban was imposed on a contaminated fish-processing plant until extensive cleaning, disinfection, a sampling scheme, and a follow-up had been performed (Gillesberg Lassen *et al.*, 2016). However, as *L. monocytogenes* kept occurring in the cold-smoked fish products, the authorities did not permit the processing plant to continue cold smoking (Gillesberg Lassen *et al.*, 2016). This exemplifies, how official control may and should intervene extensively, if the food business operator is unable to remove non-compliances.

When it comes to inspecting prevention of *L. monocytogenes*, particular attention should be paid to production quantity, process parameters, layout and maintenance of facilities, working hygiene, sanitation, sampling, and storage temperatures of processing plants (Finnish Food Authority, 2012). In Finland, if non-compliances with food safety legislation are observed during inspections, the official inspectors can give advice, demand corrections, or use coercive measures to remove them (Food Act 23/2006). The response by inspectors should reflect the type and severity of the non-compliance (EC no. 882/2004), leading to prioritizing the rapid correction of non-compliances that severely impair food safety. Non-compliances predisposing to *L. monocytogenes* contamination should be considered severe as they can contribute to foodborne outbreaks. Multiple severe non-compliances requiring coercive measures have been reported to occur in Finnish fish-processing plants (Lundén, 2013).

Although coercive measures are considered effective (Kettunen *et al.*, 2015), their use by Finnish food inspectors has, in the past, been infrequent (Jokela *et al.*, 2009). Coercive measures are still often deemed laborious and time-consuming, and uncertainty with practices and lack of guidance and routine may impede their use by some inspectors (Kettunen *et al.*, 2017b). Furthermore, inspectors have voiced concerns that the use of coercive

measures may damage their collaboration with food business operators (Kettunen *et al.*, 2017b). As the attitudes of food business operators affect their compliance and correlate with the conduct and communication by the inspector, a negotiative and cooperative approach to food inspections has been recommended (Yapp & Fairman, 2006; Läikkö-Roto & Nevas, 2014b; Kettunen *et al.*, 2017a). The Finnish Food Authority has recently started to refer to 'coaching', thereby emphasizing this customer viewpoint in official food control. Indeed, the aim of official inspections is first to guide food business operators towards food safety compliance, using enforcement only when necessary.

3 AIMS OF THE STUDY

This dissertation aimed to examine strain variability and determinants of *L. monocytogenes* tolerance towards stressors encountered in the food chain and to evaluate the current framework of *L. monocytogenes* prevention in fish-processing plants pertaining to operational practices and official food control.

Specific aims of the individual studies were as follows:

1. (i) to assess the strain variability of *L. monocytogenes* NaCl osmotic stress tolerance;

   (ii) to compile a research protocol for the data assembly and analysis of high-throughput microbial growth studies;

2. to investigate the accessory heat resistance mechanisms of *L. monocytogenes* by means of whole-genome sequence comparison;

3. to analyze the associations of fish-processing plant production, hygiene, and sanitation practices with the occurrence of *L. monocytogenes* product contamination;

4. to assess the efficacy of official food control in preventing *L. monocytogenes* fish-processing plant contamination and non-compliance.

4 MATERIALS AND METHODS

4.1 Strains and plasmids (I, II, III)

Wild-type strains of *L. monocytogenes* (n = 388) originating from several sources and belonging to the collections of the Department of Food Hygiene and Environmental Health (n = 193), University of Helsinki, and the Institute for Food Safety and Hygiene (n = 195), University of Zürich, were used in Study I. The strains represented serotypes 1/2a (n = 241), 1/2b (n = 38), 1/2c (n = 33), 3a (n = 5) and 4b (n = 71). In Study II, two *L. monocytogenes* strains AT3E and AL4E (Lundén *et al.*, 2008) were used for the comparative genomic and plasmid-mediated heat resistance studies, and *L. monocytogenes* 10403S (Bishop & Hinrichs, 1987) for horizontal gene transfer. The strains and plasmids used in the stress tolerance experiments are listed in the original studies (Study I Table 2; Study II Table 1). Furthermore, 18 isolates of *L. monocytogenes* were obtained from fish products in Study III.

4.2 Growth media (I, II)

Stock cultures were stored at -70°C in bead tubes. The *L. monocytogenes* strains were routinely grown at 37°C on blood agar, brain heart infusion (BHI) agar or BHI broth (BHIB, contains 0.5% NaCl), tryptic soy agar (TSA) or tryptic soy broth (TSB), or ALOA (Harlequin Listeria Chromogenic) agar (Labema, Helsinki, Finland). The *E. coli* strains used in the mutational analyses were grown at 37°C on Luria-Bertani (LB) agar or in LB broth (Oxoid, Cheshire, England). Suitable antibiotics (Sigma-Aldrich, St. Louis, MO, USA) and cadmium sulfate ($3CdSO4 \cdot 8H2O$) (Merck, Darmstadt, Germany) were added when required.

4.3 Typing of strains (I, II, III)

Serotyping was performed with the Listeria Antisera Set using O- and H-factor antisera (Denka Seiken, Tokyo, Japan) (I, II). Serotypes of 16 strains were additionally confirmed using multiplex-PCR (Doumith *et al.*, 2004) (I) or the Pasteur typing tool (http://bigsdb.pasteur.fr/perl/ bigsdb/bigsdb.pl?db=pubmlst_listeria_seqdef_public, accessed 13 July 2017) (II). Multilocus sequence typing (MLST) was performed with the Pasteur MLST typing tool (Haase *et al.*, 2014; Moura *et al.*, 2016) for the sequenced strains AL4E and AT3E (II). Pulsed-field gel electrophoresis (PFGE) typing of fish product

isolates was performed with the enzymes AscI and ApaI (Roussel *et al.*, 2014) (III).

4.4 Genome sequencing and comparative genomic analysis (II)

Genomic DNA from strains AL4E and AT3E was extracted (Pitcher *et al.*, 1989). Whole-genome sequencing by single-molecule real-time sequencing in the PacBio RS platform was performed at the Institute of Biotechnology (Helsinki, Finland). De novo assembly was performed using the RS_HGAP_Assembly.3 protocol (Pacific Biosciences of California, Inc., Menlo Park, CA, USA). RAST 2.0 (Aziz *et al.*, 2008) was used for genome annotation, SEED Viewer 2.0 (Overbeek *et al.*, 2005) for genome comparison, PHASTER (Dennis *et al.*, 2011; Pon *et al.*, 2016) for prophage prediction, and FGENESB (Solovyev & Salamov, 2011) for operon prediction of pLM58, a novel plasmid discovered in the heat-resistant AT3E strain. Visualizations were generated by BRIG (BLAST ring image generator) (Alikhan *et al.*, 2011) and SnapGene Viewer 3.3.4 (GSL Biotech LLC, Chicago, IL, USA). BLASTN 2.2.26 (Altschul *et al.*, 1997) was used to compare *L. monocytogenes* plasmid sequences in GenBank at National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/genome/plasmids/159?/, accessed 13 July 2017). The nucleotide sequences were deposited in the GenBank database under accession numbers CP023754 (strain AL4E), CP023752 (strain AT3E), and CP023753 (plasmid pLM58) within BioProject PRJNA412588.

4.5 Plasmid curing of pLM58 and conjugation of *clpL* (II)

Plasmid curing was performed in the heat-resistant AT3E strain on TSA plates with novobiocin (0.2 g/ml) (Margolles & de los Reyes-Gavilán, 1998). The absence of the plasmid was verified by PCR. The coding sequence of *clpL* and an upstream region including the putative promoter were conjugated into the heat-sensitive *L. monocytogenes* 10403S (kindly provided by Martin Wiedmann, Cornell University, Ithaca, NY, USA) using the PSA prophage site-specific phage integration vector pPL2 (Lauer *et al.*, 2002; Ma *et al.*, 2011). The PCR-amplified insert of the *clpL* coding sequence was ligated into the linearized pPL2 treated with recircularization-preventing Antarctic phosphatase (New England Biolabs, Ipswich, MA, USA) and propagated in *E. coli* NEBα (New England Biolabs). *E. coli* HB101 was used as the conjugation donor for the pclpL and control plasmid pPL2 without an insert, which were separately conjugated into the 10403S strain by filter mating. ALOA supplemented with 7.5 µg/ml chloramphenicol was used for the selection of transconjugants. Integration of the plasmids and presence of the insert were confirmed using specific primers. Primers used in Study II were designed

using Primer3 (v. 4.0.0) (Remm & Koressaar, 2007; Untergasser *et al.*, 2012) and are listed in Study II Table 4.

4.6 Horizontal gene transfer experiments (II)

Self-transmissibility of pLM58 was examined by standard plate mating between the strains AT3E as donor and 10403S as recipient. The strains were grown overnight at 37°C in BHI or BHI with 200 g/ml streptomycin. Diluted cultures (1:100 of BHIB) were grown to logarithmic growth phase (optical density at 600 nm, OD_{600}, of 0.5), and 100 µl of both were spotted on top of each other. After 1 h at room temperature and 24 h at 37°C, the cells were washed from the plate with BHIB. Possible transconjugants were screened after 3 days at 37°C on BHI agar with 200 µg/ml streptomycin and 65 µg/ml or 130 µg/ml CdSO4. The visible colonies were screened by PCR using specific primers.

4.7 Growth curve analyses (I, II)

Three separate colonies of each strain were individually inoculated into 5 ml (I) or 10 ml (II) of BHIB and incubated at 37°C overnight. The cultures were diluted (1:100) in fresh BHIB (II) or BHIB with NaCl 9.0% (I). The salt stress condition for Study I was chosen by piloting the growth of 20 *L. monocytogenes* strains at BHIB NaCl concentrations of 6.5%, 7.5%, and 8.5%.

Each diluted culture was loaded into separate wells of a 100-well honeycomb plate in duplicate and grown in the Bioscreen C Microbiology Reader (Growth Curves, Helsinki, Finland) at 37°C for 17 h (I) or at 42°C for 10 h (II) under continuous shaking. The OD_{600} was measured at 15-min (I) or 1-h (II) intervals. Two control strains were used in each Bioscreen experiment (I). Correspondence between the OD_{600} values and viable-cell numbers of the strains was confirmed by plate counts at mid-logarithmic and stationary growth phases (II).

Growth curve data exploration and visualization were performed using Microsoft Office Excel 2013 and R version 3.4.3 (R Core Team, 2017) (I, II). In Study I, the replicates in the outermost wells were determined as outliers and removed, and batch effects between the Bioscreen runs performed by two different laboratory technicians were normalized using the following formulas:

(1) $$\Delta_t = mean_{L_B t} - mean_{L_A t}$$

(2) $$OD_{norm} = L_A + \Delta_t$$

where $mean_{L_A}$ and $mean_L$ are the mean ODs at each time point *t* of the control strain replicates belonging to groups L_A and L_B defined by the batch (laboratory technicians A and B).

Growth parameters for each strain, including μ, λ, maximum optical density (MaxOD), and AUC, were determined with R package *grofit* (Kahm *et al.*, 2010, archived from the CRAN repository on 17 June 2018) (I, II). In Study I, the parameters were determined using Gompertz, logistic, modified-Gompertz, and Richards models, and model-free splining, all of which were compared to choose the best fit for the dataset. In Study II, the logistic model, which provided the best fit based on the Akaike information criterion (AIC), was used.

4.8 Heat resistance assay (II)

A heat survival test in 55°C water bath for 40 min was conducted on the wild-type strains 10403S, AL4E, and AT3E, cured derivative strain AT3Epc, and conjugation strains 10403SpPL2 and 10403SpclpL using the method of Lundén *et al.* (2008) with slight modifications, as presented in Study II. Strains exhibiting a $\log_{10}$ reduction of less than 1.0 $\log_{10}$ were considered heat-resistant.

4.9 Maximum growth temperatures (II)

Differences in maximum growth temperatures of the *L. monocytogenes* strains were examined with Gradiplate W10 incubator (BCDE Group, Helsinki, Finland) for 24 h at a temperature gradient of 39.2°C to 45.7°C using the method of Hinderink *et al.* (2009) with slight modifications, as presented in Study II. Growth boundaries were determined with a stereomicroscope (Olympus SZ61; Nikon, Tokyo, Japan).

4.10 Fish-processing plant investigations (III, IV)

From several geographically distinct regions in Finland, 21 fish-processing plants producing vacuum-packaged gravad or cold-smoked fish participated in Studies III and IV. Fish-processing plants A1 and A2 were considered as one fish-processing plant named A in Study III because they produced the same product: A2 performed the slicing and packaging of products processed at A1. However, they were examined as two fish-processing plants in Study IV, since separate inspection reports were available for both processing plants. Inspection reports were not available for fish-processing plant H, which was, therefore, excluded from Study IV.

4.10.1 Product sampling (III)

Between September 2014 and October 2015, a total of 425 vacuum-packaged gravad and cold-smoked fish products of the participating fish-processing plants were studied at approximately 2-month intervals. After storage at 3°C until the end of shelf life, the detection and enumeration of *L. monocytogenes* were performed at the Finnish Food Authority (known as the Finnish Food Safety Authority Evira until 31 December 2018) using International Organization for Standardization methods ISO 11290-1 and 2 (International Organization for Standardization, ISO, 2014a; International Organization for Standardization, ISO, 2014b). The API Listeria kit (bioMérieux, Marcy l'Etoile, France) was used for *L. monocytogenes* confirmation.

4.10.2 Determining the *L. monocytogenes* status of fish-processing plants (III, IV)

In Study III, if the investigated product samples of a fish-processing plant were contaminated with *L. monocytogenes* between September 2014 and October 2015, the fish-processing plant was assigned the status "*L. monocytogenes* positive", and if not, it was deemed "*L. monocytogenes* negative". In Study IV, the information extracted from the official inspection records was used to retrospectively determine the number of times *L. monocytogenes* occurred in the facilities and products of each fish-processing plant from January 2011 to December 2013.

4.10.3 Risk assessment questionnaire (III, IV)

The framework for managing *L. monocytogenes* was assessed by an in-depth inspection protocol in the format of a risk assessment questionnaire filled by the official inspectors of the participating fish-processing plants (III). The inspectors visited the fish-processing plants and returned the questionnaires in June–October 2015. The questionnaire included background information on the fish-processing plant and processing-step-specific questions concerning the following: processing environment and machinery; implementation of manufacturing processes; hygiene and sanitation practices; and opinions of the inspector on fish-processing plant compliance. It also included questions on municipal food control, i.e. the planned and executed inspections and inspections carried out during processing, the results of which were examined in Study IV. The answers were measured numerically, dichotomously, or by rating scale. The rating scales included the following: extent 1–6 (not at all – little – quite little – quite much – much – very much); frequency 1–6 (always – often – quite often – quite seldom – seldom – never); and opinion 1–4 (completely agree – somewhat agree – somewhat disagree – completely disagree). The original questions were used as such as variables or merged into summed variables.

4.10.4 Analysis of official inspection reports (IV)

Documentation covering on average 2.6 years of food control inspection reports and official microbiological sampling certificates from the years 2011–2014 was obtained from the fish-processing plants. Inspected items, including non-compliances and official control measures, were extracted from the inspection reports. The inspected items were classified into 27 sections describing the various fish-processing plant procedures, i.e., sectors of the fish-processing plant self-checking system. Each non-compliance was also assessed from the perspective of *L. monocytogenes* spread, growth, and contamination of products on a scale of "no risk" – "indirect risk" – "direct risk" based on known contamination patterns and previously reported risk factors. The efficacy of municipal official food control was additionally estimated through the fulfillment of the Finnish Food Authority requirements on the number of annual inspections (Finnish Food Authority, 2010), execution of planned inspections, and proportion of annual inspections carried out during processing.

4.11 Statistical analyses (I, II, III, IV)

Statistical analyses were performed with R version 3.4.3 (R Core Team, 2017) and International Business Machines Corporation (IBM) SPSS Statistics for Windows versions 24.0 and 25.0 (IBM, Armonk, NY, USA). Normality was tested with Kolmogorov-Smirnov or Shapiro-Wilk tests, as applicable, and by the visualization of normal Q-Q plots. In Study I, variability between replicates and strains was estimated using coefficients of variation (CV, i.e., ratio of standard deviation to mean) calculated from the respective OD_{600} values. Friedman's two-way analysis of variance by ranks was used to compare the growth parameters of different growth models and model-free splining (I). Hierarchical clustering with Ward's method by squared Euclidian distance was utilized to classify strain growth patterns (I). Kruskall-Wallis test was used to investigate the associations between serotype, growth cluster, and growth parameters (I). Independent-samples two-tailed t test was used to evaluate the differences in $\log_{10}$ reductions at 55°C, growth parameters at 42°C, and maximum growth temperatures between the *L. monocytogenes* strains (II).

Established data exploration and generalized linear modeling protocols (Zuur *et al.*, 2009; Zuur *et al.*, 2010) were followed in Studies III and IV. Collinearity was examined by Pearson and Spearman correlations and variance inflation factors. Penalized (III) or regular likelihood ratio tests (IV) were used for backward selection of variables while the residual deviance remained insignificant at the 0.05 level. Cook's distance was used to evaluate potentially influential observations (Maindonald & Braun, 2010). The explained deviance, pseudo-R^2 (Dobson, 2002), was calculated for the final models.

In Study III, the associations of the *L. monocytogenes* status of fish-processing plants with their operational procedures were examined. Cronbach's alpha of over 0.7 was used to determine summed variables. Multiplicity and sample size were considered (Schulz & Grimes, 2005; Streiner & Norman, 2011; Armstrong, 2014) by using Fisher's exact test or the Mann–Whitney U test, as applicable, to determine preliminary associations between the *L. monocytogenes* status and the individual operational practice variables, after which the variables with $p < 0.1$ were included in multivariate generalized linear modeling with the R package *logistf*. Regression coefficients were corrected for quasi-complete separation (Heinze & Schemper, 2002) using Firth's penalized method (Firth, 1993; Heinze & Ploner, 2004).

In Study IV, the number of times that *L. monocytogenes* occurred in the fish-processing plants was explained by variables consisting of percentages of non-compliances and control measures from each fish-processing plant. Generalized linear modeling was carried out using R package *glm* (family = 'poisson' and 'quasipoisson', link = 'log'). Overdispersion was tested with *AER* package (Kleiber & Zeileis, 2008).

5 RESULTS

5.1 *L. monocytogenes* stress tolerance (I, II)

A high-throughput data assembly and analysis protocol (I) and comparison of whole-genome sequences (II) were used to determine enhanced growth and survival of *L. monocytogenes* under stress conditions. Large strain variability, serotype-dependent associations, and a novel genetic mechanism of stress tolerance were uncovered.

5.1.1 Strain variability of NaCl stress tolerance (I)

The compiled data assembly and analysis protocol for high-throughput microbial growth experiments included the following steps (summarized in Study I Table 1): measurement of growth ability under stress (step 1), selection of a suitable method for growth parameter calculation (step 2), comparison of growth patterns between strains (step 3), and biological interpretation of the discovered differences (step 4). NaCl 9.0% was chosen as the salt stress condition, as the piloted strains exhibited negligible differences in growth at NaCl 6.5% and 7.5%, whereas NaCl 8.5% began to moderately increase the lag time and decrease the growth rate and maximum optical density of some strains. After a careful inspection and removal of outliers and batch effects (Section 4.7), the Bioscreen experiments of the 388 *L. monocytogenes* strains exhibited strain-specific and serotype-dependent variability of growth patterns at NaCl 9.0% (Fig. 4).

Notable differences were discovered between the growth parameter calculation methods in their suitability to model *L. monocytogenes* growth at NaCl 9.0% (Study I Table 3 and Study I Fig. 1). Model-free splining (Kahm *et al.*, 2010) was deemed to best fit this dataset. The strain variability of growth patterns was assessed by the visual inspection of growth curves and comparison of alternative hierarchical clustering outcomes utilizing different variables (Study I Figs. S12–S15). Using the spline parameter AUC as the clustering variable, the classification of similarly growing strains into clusters of “good” (n = 182), “average” (n = 116), and “poor” (n = 90) growth enabled the identification of NaCl osmotolerant and susceptible *L. monocytogenes* strains (Fig. 4).

Of the serotype 4b strains, 97% clustered in “good”, and of the 1/2b strains, 45% clustered in “good” and 50% in “average”. Of the serotype 1/2a and 1/2c strains, 60% and 97%, respectively, clustered in “poor”. These differences in the proportions of serotypes in the growth clusters were statistically significant (z-test, $p < 0.05$). Similarly, the spline growth parameters differed significantly from one another between most growth clusters and serotypes (Kruskall-Wallis test, $p < 0.03$) (Fig. 5).

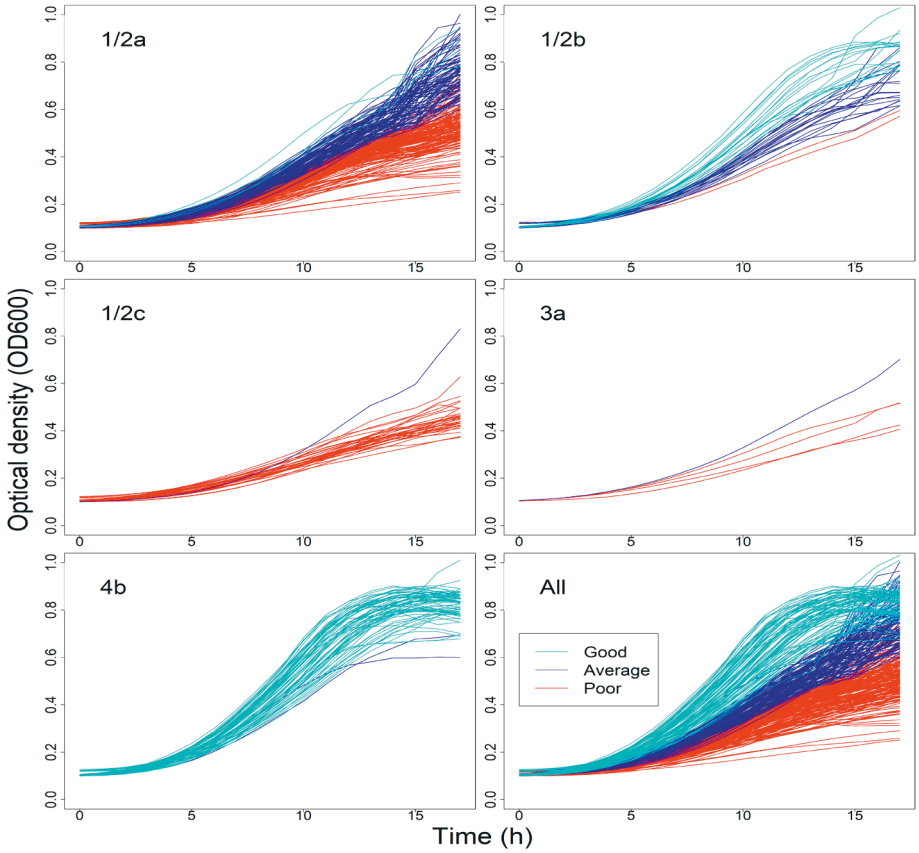

Figure 4 *Listeria monocytogenes* strains (n = 388) by serotype and growth cluster in NaCl 9.0%. Strain growth curves belonging to serotypes 1/2a, 1/2b, 1/2c, 3a, 4b, and all serotypes are shown. Division into growth clusters is visualized by color as indicated in the insert. Adopted from Study I.

5.1.2 Genomic comparison of heat-resistant and heat-sensitive strains (II)

The heat-resistant *L. monocytogenes* strain AT3E and heat-sensitive AL4E were of the same serotype, 1/2c, and multilocus sequence type, ST9. In genome comparison, the heat-resistant strain exhibited 49 and the heat-sensitive strain 21 unique chromosomal genes, most of which were hypothetical or phage-related. Additionally, the heat-resistant strain harbored a novel 58-kb plasmid, designated pLM58. Plasmid annotation exposed a putative open reading frame (ORF) encoding an ATP-dependent Clp protease ATP-binding subunit (ClpL), which shared 98% nucleotide sequence identity (E value of 0.0) with clpL2 of *Lactobacillus rhamnosus* and 46% amino acid identity with plasmid-borne clpK2 of *Escherichia coli* (E value of 9E–175). pLM58 carried similar genes to cadAC that mediate cadmium resistance in *Staphylococcus aureus*.

μ	1/2b	1/2c	3a	4b	Average	Good	λ	1/2b	1/2c	3a	4b	Average	Good
1/2a	0.03*	<0.001*	1.0	<0.001*			**1/2a**	1.0	<0.001*	1.0	1.0		
1/2b		<0.001*	0.1	0.1			**1/2b**		<0.001*	1.0	1.0		
1/2c			1.0	<0.001*			**1/2c**			1.0	0.001*		
3a				0.002*			**3a**				1.0		
Poor					<0.001*	<0.001*	**Poor**					<0.001*	<0.001*
Average						0.2	**Average**						<0.001*
MaxOD	1/2b	1/2c	3a	4b	Average	Good	**AUC**	1/2b	1/2c	3a	4b	Average	Good
1/2a	<0.001*	<0.001*	1.0	<0.001*			**1/2a**	<0.001*	<0.001*	0.9	<0.001*		
1/2b		<0.001*	0.007*	0.6			**1/2b**		<0.001*	0.001*	0.03		
1/2c			1.0	<0.001*			**1/2c**			1.0	<0.001*		
3a				<0.001*			**3a**				<0.001*		
Poor					<0.001*	<0.001*	**Poor**					<0.001*	<0.001*
Average						<0.001*	**Average**						<0.001*

Figure 5 Kruskall-Wallis test p-values for associations of each spline growth parameter between the tested *Listeria monocytogenes* strains (n = 388) of serotypes 1/2a, 1/2b, 1/2c, 3a, and 4b, and of the growth clusters "Poor", "Average", and "Good", respectively, at NaCl 9.0%. Significant associations are indicated with an asterisk. μ corresponds to growth rate (OD_{600} units/h), λ to lag time (h), MaxOD to maximum growth (OD600 units), and AUC is area under curve calculated by integral. Adopted from Study I.

5.1.3 Determinants of heat resistance (II)

At 55°C, the strain AT3E was more heat resistant than AL4E (0.0 CFU/ml vs. 1.4 CFU/ml log10 reduction; $p < 0.01$) (Fig. 6). The curing of pLM58 from the heat-resistant strain resulted in significant reduction of heat resistance of the cured derivative strain AT3Epc at 55°C (log10 reduction of 1.1 CFU/ml, $p < 0.001$) (Fig. 6). Correspondingly, the introduction of clpL into the heat-sensitive *L. monocytogenes* 10403S in the pPL2 backbone increased its heat resistance at 55°C (log10 reduction from 1.2 CFU/ml to 0.4 CFU/ml ($p < 0.01$) (Fig. 6). Conversely, the conjugation of the control plasmid pPL2 alone did not affect heat resistance (Fig. 6). The maximum growth temperature of the heat-sensitive strain was 0.5°C higher than that of the heat-resistant strain ($p < 0.01$). No significant differences were observed in the maximum growth temperature and kinetic growth parameters at 42°C between the parent, derivative, and conjugated strains.

Upon plate-mating, (i) cadmium resistance (Lebrun *et al.*, 1994; McLauchlin *et al.*, 1997) and (ii) streptomycin resistance (den Bakker *et al.*, 2012) were used to facilitate the selection of (i) recipient cells without pLM58 and (ii) possible transconjugants. pLM58 was determined to be putatively nonconjugative, as the AT3E donor and 10403S recipient strains grew on positive-control plates, but not on selective plates containing both 130 µg/ml $CdSO_4$ and 200 µg/ml streptomycin. In addition, the known type IV secretion system genes required for the conjugation of self-transmissible plasmids (Teuber et al., 2003; Smillie et al., 2010; Goessweiner-Mohr et al., 2014) were missing from pLM58.

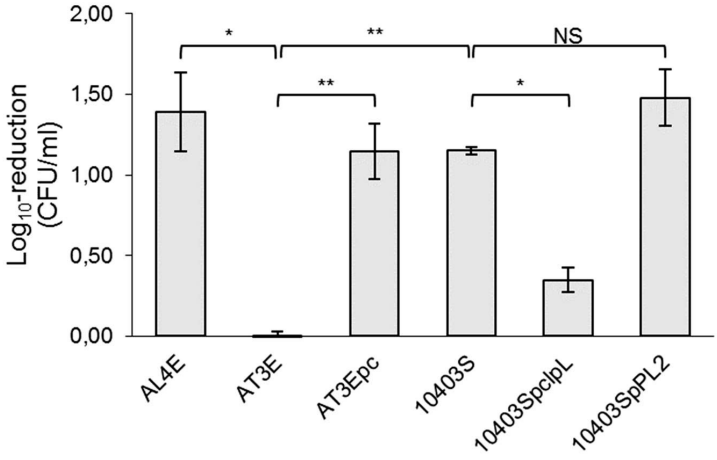

Figure 6 Susceptibility of wild-type and modified *Listeria monocytogenes* strains to heat stress at 55°C after 40 minutes. Log10-reduction values represent the mean values and error bars the standard deviations of three replicate cultures. Significance was determined by independent samples 2-tailed t test (*, p < 0.01; **, p < 0.001; NS, not significant).

5.2 *L. monocytogenes* occurrence in fish products and processing plants (III, IV)

Based on the analyses of official inspection records, *L. monocytogenes* occurred 1 to 14 times in the products or facilities of 9/21 fish-processing plants, while it did not occur at all in 12/21 fish-processing plants, in 2011–2013 (Table 5). During the years fish-processing plants A1, B, and D suffered from recurrent *L. monocytogenes* contamination in products and on food contact surfaces, whereas the contamination of fish-processing plants E, F, and I–L was sporadic and rarely observed in products or on food contact surfaces.

In 2014–2015, 4.2% of the sampled vacuum-packaged gravad and cold-smoked fish products had *L. monocytogenes* contamination (10–20 cfu/g). Contamination only occurred in sliced products (6.2%) and was significantly more common among "sliced, gravad" than "sliced, cold-smoked" products (11% vs. 1.9%, respectively, Fisher's exact test, p = 0.001). The *L. monocytogenes* positive product samples originated from 7/21 fish-processing plants (Table 5), and their *L. monocytogenes* isolates displayed 10 different PFGE pulsotypes. Contamination recurred in the products of fish-processing plants A, B, and E, with the original pulsotype reappearing in each plant on separate sampling occasions (Table 5). Fish-processing plants A1, B, and D–F presented with *L. monocytogenes* contamination in both Studies III and IV (Table 5).

5.3 Fish-processing plant practices associated with *L. monocytogenes* product contamination (III)

The number of processing machines correlated with the fish-processing plant output in tons (Spearman's rho 0.8, p < 0.001) and was associated with *L. monocytogenes* product contamination (Table 6). Products were only contaminated in the fish-processing plants using skinning and slicing machines. Staff movement between processes from areas of low to high hygiene during the production day was associated with an increased risk of product contamination in fish-processing plants using a slicing machine (Table 6). During the processing day in-between-process cleaning for the slicing machine was performed by 12 but not by 3 fish-processing plants, of which 33% and 100%, respectively, were *L. monocytogenes* positive (Fisher's exact test, p = 0.08; penalized logistic regression chi-squared = 0.5, p = 0.5). Rinsing with water as an in-between-process cleaning method was mentioned by 7 but not by 9 fish-processing plants, of which 57% and 22%, respectively, were *L. monocytogenes* positive (Fisher's exact test, p = 0.3). Disinfection was mentioned as part of the in-between-process cleaning protocol by 5 but not by 11 fish-processing plants, of which 0% and 55%, respectively, were *L. monocytogenes* positive (Fisher's exact test, p = 0.09). Cleaning of facilities

Table 5 Occurrence of non-compliances (NCs) and *Listeria monocytogenes* (*Lm*) in the investigated fish-processing plants (FPPs) according to their inspection records for 2011–2014 (IV), and occurrence of *Lm* in the vacuum-packaged, ready-to-eat (RTE) gravad and cold-smoked fish products in 2014–2015 (III). Adopted from Studies III and IV.

FPP	Total production[a]	No. of inspection reports (IV)	% of NCs from inspected items	% NCs assessed to pose a risk of *Lm* spread, growth, or contamination	No. of *Lm*-positive samples[b] in IV (of which *Lm*-positive RTE products)	Type of *Lm* contamination (IV)	No. of sampled packages (III)	No. of *Lm* positive packages (%) (III)	Same *Lm* PFGE type on separate sampling occasions (III)
A1*	Medium	13	68	66	14 (2)	recurrent	21	5 (24)	yes
A2*	-	7	50	62	0 (0)	none	-	-	-
B	Large	16	69	68	8 (2)	recurrent	18	3 (17)	yes
C	Medium	5	23	33	0 (0)	none	21	3 (14)	no
D	Large	13	59	80	8 (3)	recurrent	21	2 (9.5)	no
E	Large	17	26	55	2 (1)	sporadic	21	2 (9.5)	yes
F	Medium	13	29	35	2 (0)	sporadic	21	2 (9.5)	no
G	Large	16	67	42	0 (0)	none	21	1 (4.8)	no
H[c]	Small	-	-	-	-	-	17	0 (0)	no
I	Medium	6	71	53	5 (0)	sporadic	21	0 (0)	no
J	Medium	24	61	64	3 (0)	sporadic	21	0 (0)	no
K	Large	27	38	59	2 (1)	sporadic	21	0 (0)	no
L	Medium	5	56	67	2 (1)	sporadic	21	0 (0)	no
M	Large	10	39	61	0 (0)	none	21	0 (0)	no
N	Medium	17	50	69	0 (0)	none	21	0 (0)	no
O	Medium	9	45	60	0 (0)	none	21	0 (0)	no
P	Small	2	33	43	0 (0)	none	12	0 (0)	no
Q	Small	5	15	29	0 (0)	none	21	0 (0)	no
R	Small	5	41	56	0 (0)	none	21	0 (0)	no
S	Small	7	63	61	0 (0)	none	21	0 (0)	no
T	Small	4	39	53	0 (0)	none	21	0 (0)	no
U	Small	5	64	39	0 (0)	none	21	0 (0)	no

*FPPs A1 and A2 produced the same product and were handled as two FPPs in 2011–2013 (IV) and as one FPP, named A, in 2014–2015 (III).; [a]Large > 1 000 000 kg/year; medium = 100 000–1 000 000 kg/year; small < 100 000 kg/year; [b]Samples were collected at approximately 2-month intervals from each FPP during a 14-month period.; [c]Inspection records were not obtained from FPP H.

during processing was performed by 6 but not by 15 fish-processing plants, of which 50% and 27%, respectively, were *L. monocytogenes* positive (Mann-Whitney U test $p = 0.4$).

On average, periodic thorough sanitation was performed more often at *L. monocytogenes*-negative than -positive fish-processing plants both for the processing environment (14 vs. 3 times/year; Mann–Whitney U test, p = 0.05) and the machinery (10 vs. 5 times/year; Mann–Whitney U test, p = 0.4). The multivariate analyses illustrated that the more often a periodic thorough sanitation of the processing environment was executed, the smaller the risk of the fish-processing plant exhibiting *L. monocytogenes* product contamination (Table 6). Furthermore, periodic thorough sanitation of the vacuum machine, which co-occurred with the periodic thorough sanitation of the slicing machine (Pearson's correlation coefficient 0.6, p = 0.01), was associated with a decreased risk of *L. monocytogenes* product contamination (Table 6).

5.4 Fish-processing plant compliance (III, IV)

A total of 1456 non-compliances had been documented in 2011–2014 in the official inspection reports of the fish-processing plants evaluated (IV). Most fish-processing plants (81–90%) had non-compliances in the following sectors of the fish-processing plant self-checking system: sanitation, processing hygiene, maintenance, food hygiene sampling and samples, orderliness, temperature control, or suitability of premises and equipment.

5.4.1 Opinions on compliance (III)

The opinions of the inspectors concerning food safety compliance of the fish-processing plants were inquired in the risk-assessment questionnaire in 2015 (III). The inspectors opined that hands and utensils were "often" to "quite often" washed and gloves changed as required in the fish-processing plants. On average, "little" dirtiness or erosion of processing surfaces was estimated to occur. The inspectors agreed somewhat less that the fish-processing plant conformed to official control in *L. monocytogenes*-positive than -negative fish-processing plants (Whitney U test, $p = 0.1$). Problems in collaboration between the fish-processing plant and the inspector had only been recorded in the inspection reports of fish-processing plants A, B, and D; these plants had recurrent *L. monocytogenes* contamination (Table 5).

5.4.2 Non-compliances associated with *L. monocytogenes* occurrence in fish-processing plants (IV)

According to generalized linear modeling, the occurrence of *L. monocytogenes* increased in the facilities and products of fish-processing plants during 2011–2013 when larger proportions of the fish-processing plant non-compliances

recurred, concerned processing machinery, and did not result in official control measures (Table 6). Head removal, filleting, and trimming lines were documented in the inspection reports as particularly problematic areas for *L. monocytogenes* control. Recurrent deficiencies in cleanliness and condition of surfaces and *L. monocytogenes* contamination of processing machines, were documented by inspectors in fish-processing plants A, B, and D (Table 5).

In listeria-positive and listeria-negative fish-processing plants, 61% and 51% of the non-compliances, respectively, were estimated to potentially pose an "indirect" or "direct" listeria risk (Student's t-test, $p = 0.09$). Of the non-compliances concerning processing hygiene, food hygiene sampling and samples, sanitation, suitability of premises and equipment, working hygiene, and wastewater management, 21–35% were estimated to cause "direct" listeria risk.

5.4.3 Inspections and official control measures (IV)

Of the inspections planned for 2014, all had been executed in 5/21 fish-processing plants. Inspection during production appeared a more common practice in listeria-positive than listeria-negative fish-processing plants (82% vs. 51%; Mann-Whitney U test, $p = 0.08$). Of all recorded non-compliances ($n = 1456$), 78% were followed by demands for correction, 11% by advice without demands, 9% by no requests for control measures, and 0.1% by coercive measures. The inspectors had more seriously considered the use of coercive measures in the official control of the fish-processing plants with *L. monocytogenes* product contamination (Mann–Whitney U test, $p = 0.06$). However, coercive measures pertaining to *L. monocytogenes* control were not used during the investigated period in fish-processing plants A, B, and D (Table 5), which had the most widespread contamination. Mention of re-inspection was recorded for 65% of the non-compliances, of which 51% were documented to have been completely and 18% partially corrected. During the study period 33% of the non-compliances provably recurred.

Non-compliances posing either an "indirect" or "direct" listeria risk received more demands for correction than other non-compliances (80% vs. 74%; Fisher's exact test, $p = 0.007$), but no difference was observed in their correction (72% vs. 73% completely or partially corrected, respectively; Fisher's exact test, $p = 0.8$). The time limits given for the correction of non-compliances shortened according to the estimated listeria risk (time limit medians "no risk" 30 days, "indirect" 13 days, "direct" 0 days; Kruskall-Wallis, $p < 0.001$), but no differences were observed between the correction of non-compliances and the presence or absence of time limits (73% vs. 71% completely or partially corrected, respectively; Fisher's exact test, $p = 0.5$).

Table 6. Results of three (a, b, and c) separate generalized linear models (a and b penalized logistic regression; c quasi-Poisson regression) for variables associated with (a and b) the occurrence of *Listeria monocytogenes* in the products of fish-processing plants (FPPs) and (c) increase in *L. monocytogenes* occurrence in FPPs. Adopted from Studies III and IV.

	Model attribute or parameter	Deviance	Df	Chi-squared	F test	*p* value	*β*	SE for *β*	OR	CI 95%
a.										
	Null deviance	16.2	20							
	Residual deviance	5.6	18	10.6		0.005				
	Intercept					0.004	–5.18	2.83		
	Periodic thorough sanitation for the processing environment (times/year)		1	6.4		0.01	–0.11	0.059	0.90	0.71–0.98
	Number of processing machines		1	8.9		0.003	1.91	1.01	6.7	1.6–1730
b.										
	Null deviance	19.6	14							
	Residual deviance	10.5	12	9.1		0.01				
	Intercept					0.01	–2.72	1.53		
	Periodic thorough sanitation for vacuum machine (yes vs. no)		1	4.3		0.04	2.70	1.63	15	1.03–2060
	Staff movement from areas of low towards high hygiene during production day (no vs. yes)		1	4.5		0.03	2.71	1.61	15	1.1–2100
c.										
	Null deviance	97.7	20							
	Residual deviance	34.2	17		63.5	< 0.001				
	Intercept						6.63	3.71		
	% of non-compliances related to processing machinery		1		26.4	< 0.001	0.21	0.05		
	% of non-compliances given an official control measure by inspector		1		6.48	0.02	-0.13	0.05		
	% of non-compliances recurred		1		15.1	0.001	0.11	0.03		

a. All FPPs from Study III (n = 21, pseudo-R^2 = 65%); b. FPPs with slicing machine from Study III (n = 15, pseudo-R^2 = 46%); c. All FPPs from study IV (n = 21, pseudo-R^2 = 65%); Df = degrees of freedom; β = estimate; SE = standard error; OR = odds ratio; CI = confidence interval

6 DISCUSSION

6.1 Determining enhanced stress tolerance of *L. monocytogenes*

The growth of *L. monocytogenes* lineage I strains was better than that of lineage II strains under NaCl osmotic stress. Overall, strain variability of stress tolerance appeared to be large for *L. monocytogenes*, and many strains were able to acclimate to the most extreme saline conditions occurring in the food chain. The novel discovery of heat resistance-mediating *clpL* carried by plasmid pLM58 illustrated that the mechanisms underlying strain variability of stress tolerance can be accessory genetic traits carried by mobile genetic elements. Our results imply that plasmid-borne stress resistance genes could contribute to enhanced survival of *L. monocytogenes* strains under conditions encountered in food-processing environments.

6.1.1 Lineage associations and strain variability of *L. monocytogenes* at NaCl stress

At NaCl 9.0%, *L. monocytogenes* strains of serotypes 1/2b and 4b of lineage I grew significantly better than serotypes 1/2a, 1/2c, and 3a of lineage II, implying that the ability to grow under NaCl osmotic stress differs between these phylogenetic lineages. Osmotolerance of *L. monocytogenes* has been linked to its virulence (Rouquette *et al.*, 1996; Gaillot *et al.*, 2000; Chaturongakul *et al.*, 2008; Watson *et al.*, 2009; Payne *et al.*, 2013), and strains of lineage I typically possess virulence potential, whereas attenuated virulence has been described for many lineage II strains (Norton *et al.*, 2001b; Jacquet *et al.*, 2004; Moura *et al.*, 2016). Our findings of enhanced osmotolerance in lineage I, isolates of which often cause human listeriosis (Orsi *et al.*, 2011), endorse these previous observations. However, lineage II strains are also affiliated with listeriosis cases (Lukinmaa *et al.*, 2003; Parihar *et al.*, 2008; Lopez-Valladares *et al.*, 2018), occur in RTE food products (Lopez-Valladares *et al.*, 2018), and have been hypothesized to have enhanced tolerance towards environmental conditions in food-associated environments (Orsi *et al.*, 2011; Ribeiro & Destro, 2014). None of the tested *L. monocytogenes* strains were completely inhibited by NaCl 9.0%, a condition encountered in the food chain mainly in brining solutions. Based on our observations of lineage-associated NaCl tolerance, high salinity environments could provide a growth niche, particularly for *L. monocytogenes* strains of lineage I.

Selecting an appropriate level of stress to examine the strain variability of *L. monocytogenes* stress tolerance was deemed essential in this study. In previous studies on strain variability, NaCl concentrations of 8% and below

have been utilized (Vialette *et al.*, 2003; Bergholz *et al.*, 2010; Magalhães *et al.*, 2016; Hingston *et al.*, 2017), but in our dataset, differences between the growth patterns of *L. monocytogenes* strains only became apparent at the relatively high NaCl concentrations of 8.5–9.0% at 37°C. Although many *L. monocytogenes* strains appear highly tolerant towards NaCl osmotic stress, the overall stress response depends on other environmental exposures, such as temperature, pH, water activity, nutrients, and sanitizing agents (Van Der Veen *et al.*, 2008; Vogel *et al.*, 2010; Ferreira *et al.*, 2014; Magalhães *et al.*, 2016). In complex food systems, the *L. monocytogenes* growth patterns described here for NaCl osmotic stress may change depending on other environmental factors, including low temperature. Nevertheless, as illustrated by the clusters "poor", "average", and "good", the tested *L. monocytogenes* strains exhibited notable variability in growth at NaCl osmotic stress, implying that the reservoir of growth ability within the species is large. This may help the population to employ a strategy called bet-hedging (Veening *et al.*, 2008), where it acclimates to differing environmental conditions, conquering various habitats, including food-related environments.

6.1.2 Plasmid-mediated heat resistance

We reported, for the first time, plasmid-mediated heat resistance of *L. monocytogenes*. Plasmids have been known to contribute to *L. monocytogenes* resistance towards antibiotics (Poyart-Salmeron *et al.*, 1990; Hadorn *et al.*, 1993), disinfectant (Elhanafi *et al.*, 2010; Jiang *et al.*, 2016; Kremer *et al.*, 2017), and heavy metals (Lebrun *et al.*, 1992; Lebrun *et al.*, 1994), but evidence on thermal resistance has been scarce. The presence of plasmids has previously been associated with cold sensitivity of *L. monocytogenes* (Hingston *et al.*, 2017). Decreased heat survival of *L. monocytogenes* following plasmid curing has recently been reported (Naditz *et al.*, 2019). Furthermore, annotations related to heat stress have been discovered among *L. monocytogenes* plasmids (Fox *et al.*, 2016).

Our whole-genome sequence comparison of a heat-resistant and a heat-sensitive *L. monocytogenes* strain led to the discovery of the plasmid pLM58, and within it, the ORF annotated *clpL*, encoding the ATP-dependent protease ClpL. Clp ATPases include heat shock proteins acting as chaperones involved in the virulence and stress tolerance of *L. monocytogenes* (Rouquette *et al.*, 1996; Gaillot *et al.*, 2000; Nair *et al.*, 2000). Plasmid curing and conjugative analyses yielded phenotypic evidence of increased heat resistance of *L. monocytogenes* due to the plasmid-borne *clpL*. This mechanism has not been described before in *L. monocytogenes*, but upregulation of *clpL* from an *L. rhamnosus* plasmid during heat shock has been reported (Suokko *et al.*, 2005), indicating that plasmid-borne *clpL* may have a universal role in heat resistance of Gram-positive bacteria. *clpL* of pLM58 was found to moderately resemble plasmid-borne *clpK* of the Gram-negative *E. coli*, and thermotolerance linked to an ATPase-encoding *clpK* gene has been noted in

an *E. coli* dairy isolate (Boll *et al.*, 2017) and a nosocomial *Klebsiella pneumoniae* strain (Bojer *et al.*, 2010). Furthermore, upregulation of plasmid-borne *clpL* has recently been described in *L. monocytogenes* under NaCl and acid stress (Hingston *et al.*, 2019a), implying its involvement in several *L. monocytogenes* stress responses.

In our experiments, plasmid-borne *clpL* was associated with heat survival at 55°C, but not with maximum growth temperatures or growth ability at 42°C. Conversely, chromosomal *clpC* and *clpP* play a role in the growth of *L. monocytogenes* under several stresses, including NaCl and temperatures of 42–43°C (Rouquette *et al.*, 1996; Gaillot *et al.*, 2000). This suggests that tolerance mechanisms to thermal kill are different from those allowing growth at the high end of the growth temperature range – a phenomenon also described for *K. pneumoniae* (Bojer *et al.*, 2010). Similarly, acid survival of *L. monocytogenes* has been associated with reduced growth at mild acid stress (Metselaar *et al.*, 2013). Such heterogeneity of stress tolerance may benefit cell survival under differing stress conditions (Metselaar *et al.*, 2013) and contribute to bet-hedging (Veening *et al.*, 2008). Plasmids that harbor genes for environmental fitness, virulence, and antibiotic resistance (Bojer *et al.*, 2010; Gullberg *et al.*, 2014; Fox *et al.*, 2016) may, thereby, enhance the ability of plasmid-harboring pathogens to occupy different habitats. Given that plasmid harborage appears to be more common among recurrent than sporadic *L. monocytogenes* isolates from food processors (Harvey & Gilmour, 2001), plasmid-mediated stress resistance may contribute to enhanced survival of *L. monocytogenes* in food-processing environments.

6.1.3 Methodologies to study stress tolerance

Our systematic assembly and analysis protocol of growth data derived from absorbance measurements enabled the investigation of *L. monocytogenes* osmotic stress tolerance diversity with an unprecedented scope. The affirmation of some previously described serotype- and lineage-dependent osmotic stress phenomena (Van Der Veen *et al.*, 2008; Bergholz *et al.*, 2010; Ringus *et al.*, 2012; Ribeiro & Destro, 2014) confirms the suitability of our protocol for *L. monocytogenes* stress tolerance investigations. As our aim was to compare growth curves and kinetic parameters proportionate to one another, careful data exploration and appropriate growth parameter estimation were essential to achieve comparability throughout the dataset. Technical variation was resolved by removing outliers and performing a normalization procedure.

Contrary to earlier studies (Dalgaard & Koutsoumanis, 2001; López *et al.*, 2004; Pla *et al.*, 2015), none of the tested growth models adequately described our dataset, but model-free splining (Kahm *et al.*, 2010) provided the best fit. The spline parameter AUC was able to differentiate ordinal growth patterns when used in hierarchical clustering, as AUC can summarize growth patterns

when λ increases while μ and MaxOD decrease in an ordinal fashion between strains. However, AUC cannot differentiate strains displaying non-ordinal growth patterns. Therefore, the overall variability in the shape of the growth curves should be considered when choosing a method to classify microbial growth patterns. Although the observed overlap of the clusters "poor", "average", and "good" indicated that growth at NaCl osmotic stress is a continuous phenotype, the clusters were deemed to meaningfully represent the differing growth patterns, as they captured biological variability of *L. monocytogenes* serotypes.

The observations on strain-specific growth variation raise questions about the genetic mechanisms underlying the differing stress tolerance phenotypes. In Study II, a relatively simple comparison of the whole-genome sequences of a resistant and a sensitive *L. monocytogenes* strain produced the discovery of a novel accessory mechanism of heat stress resistance. Correspondingly, large-scale characterization of tolerant and susceptible strains by the methodologies of Study I paves the way for the utilization of whole-genome sequencing and complex bioinformatics pipelines, such as sequence element enrichment analysis (SEER, Lees *et al.*, 2016), to investigate underlying genotypes of stress tolerance phenotypes. For such pipelines, sequencing technologies that yield contiguous sequences (Illumina platform) currently suffice. However, a closed genome was required in Study II to differentiate the plasmid sequence from the chromosome, and, thus, the PacBio RS platform (Section 4.4) was utilized.

6.2 Characterizing *L. monocytogenes* contamination in fish-processing plants

In 2011–2013, sporadic *L. monocytogenes* contamination was documented in fish-processing plants E, F, and I–L and recurrent contamination in fish-processing plants A1, B, and D, while no contamination was recorded in the other fish-processing plants (Table 5). In 2014–2015, *L. monocytogenes* only occurred in sliced products of 7/21 fish-processing plants, all of which had slicing and skinning machines, and the *L. monocytogenes* product contamination recurred in fish-processing plants A, B, and E (Table 5). Consequently, fish-processing plants A1 and B exhibited recurrent *L. monocytogenes* contamination throughout several years. *L. monocytogenes* occasionally inevitably comes with raw materials into the fish-processing facilities (Eklund *et al.*, 1995; Huss *et al.*, 2000; Gudmundsdottir *et al.*, 2005; Markkula *et al.*, 2005), which then serve as the major site where product contamination occurs and where it can also be eliminated (Autio *et al.*, 1999; Lappi *et al.*, 2004b; Markkula *et al.*, 2005; Miettinen & Wirtanen, 2006). We found associations between the number of processing machines and *L. monocytogenes* fish product contamination (III), and the proportion of processing machine-related non-compliances and *L. monocytogenes* occurrence in fish-processing plants (IV).

Recurrent *L. monocytogenes* contamination predominantly ensues from persistent contamination of processing environments and machinery (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Dauphin *et al.*, 2001; Norton *et al.*, 2001a; Vogel *et al.*, 2001b; Lundén *et al.*, 2002; Hoffman *et al.*, 2003; Lundén *et al.*, 2003b; Autio *et al.*, 2004; Keto-Timonen *et al.*, 2007; Malley *et al.*, 2013). Judging by the inspection reports, dirty processing machinery in poor condition indisputably aggravated the recurrent contamination of fish-processing plants A1, B, and D in 2011–2013. Persistent contamination was also likely behind the recurrent contamination of the fish-processing plants in 2014–2015, which is supported by the recurrence of *L. monocytogenes* PFGE pulsotypes. However, the overall low occurrence of *L. monocytogenes* observed in the product samples implies that the transfer of *L. monocytogenes* from the facilities to the products may have been rare. Additionally, the low cell counts of *L. monocytogenes* might have been due to a low level of initial contamination. Nevertheless, it should be noted that *L. monocytogenes* takes several days to grow at 3°C (Markkula *et al.*, 2012a; Pöntinen *et al.*, 2015) and not all strains have equal cold tolerance (Arguedas-Villa *et al.*, 2014), which may also have influenced the detection of only low levels of product contamination.

6.3 Improving *L. monocytogenes* preventive measures at fish-processing plants

Based on our findings, preventing *L. monocytogenes* contamination of fish-processing environments and RTE fish products requires enhanced sanitation, hygiene, and management of non-compliances, in which measures taken by both the fish-processing plants and their official control play a crucial role.

6.3.1 Strengthening sanitation and hygiene measures

An increased number of processing machines – particularly skinning and slicing machines – likely explains why *L. monocytogenes* contamination only occurred in the large and medium-sized fish-processing plants, as these two machines were absent from the small fish-processing plants. Complex machines are known to provide niches for *L. monocytogenes* contamination (Autio *et al.*, 1999; Vogel *et al.*, 2001a; Lundén *et al.*, 2002; Thimothe *et al.*, 2004; Gudmundsdottir *et al.*, 2005; Nakamura *et al.*, 2006; Chitlapilly Dass *et al.*, 2010; Di Ciccio *et al.*, 2012). Although the cleanliness of machines is essential for mitigating food safety threats (Autio *et al.*, 1999; Huss *et al.*, 2000; Lundén *et al.*, 2002; Tompkin, 2002; Nakamura *et al.*, 2006; Tolvanen *et al.*, 2009; Blatter *et al.*, 2010; Bērziņš *et al.*, 2010), poor hygienic design can hinder proper sanitation (Aarnisalo *et al.*, 2006; Giske *et al.*, 2017).

We found that infrequent periodic thorough sanitation of the processing environment and lack of periodic thorough sanitation of the vacuum machine

– and correspondingly, the slicing machine – increased the risk of *L. monocytogenes* product contamination. The implementation of a periodic thorough sanitation protocol might encourage more meticulous cleaning and dismantling of complex machinery than during everyday sanitation, which may assist in the maintenance of adequate cleanliness for *L. monocytogenes* prevention. This assumption is supported by a recent survey concluding that implementation of a thorough sanitation event increased self-reported commitment to food safety (Wu *et al.*, 2020). Additionally, our results imply that in-between-process cleaning of machinery might support the prevention of *L. monocytogenes* contamination if combined with disinfection; by contrast, mere rinsing with water might aggravate contamination. Water rinses and cleaning while processing are considered poor hygienic practices (Tompkin, 2002; Lappi *et al.*, 2004b), yet, our results show that they are performed by fish-processing plants. Overall, our findings reveal the need to enhance self-checking system efforts on cleanliness, maintenance, and hygienic handling of processing machines to reduce *L. monocytogenes* occurrence in fish-processing plants.

The risk of product contamination with *L. monocytogenes* increased with staff movement from processing areas of low hygiene to high hygiene during a working day. This was significant in the fish-processing plants using a slicing machine, which were large or medium-sized fish-processing plants, where processing facilities would also have been more complex than in small fish-processing plants. Job rotation during the processing day and poor compartmentalization of processing lines have been associated with *L. monocytogenes* contamination (Rørvik *et al.*, 1997; Lundén *et al.*, 2003b; Keto-Timonen *et al.*, 2007). Infrastructure and resources that support hygienic practices can enhance the prevention of *L. monocytogenes* (Clayton *et al.*, 2002; Hicks *et al.*, 2004). Thus, careful attention should be paid to *L. monocytogenes* risks when designing processing facilities and hygienic routes.

6.3.2 Management of non-compliances

Non-compliances commonly occurred in the fish-processing plants and most were estimated to predispose to *L. monocytogenes*. Previously, Finnish fish industry operators have considered legislative requirements pertaining to self-checking systems, layout and transport routes, and package labeling especially difficult to implement (Tähkäpää *et al.*, 2009). In our investigation, however, non-compliances were particularly common in the sectors of sanitation, processing hygiene, maintenance, food hygiene sampling, orderliness, temperature control, and suitability of premises and equipment. This implies some inconsistency between the practice and perception of food safety, as Finnish fish-processing plants have also stated that they correct the issues brought forward by inspectors (Kettunen *et al.*, 2017b) and regard the risks of their operations as smaller than other food processors (Nevas *et al.*, 2013).

Although non-compliances predisposing to *L. monocytogenes* were somewhat more frequent in *L. monocytogenes*-positive than -negative fish-processing plants, the occurrence of such non-compliances in all fish-processing plants indicates that the means of responding to them may contribute to the observed differences in *L. monocytogenes* occurrence. The inspectors opined that food safety compliance might have been somewhat poorer in *L. monocytogenes*-positive than -negative fish-processing plants. Additionally, fish-processing plants A1, B, and D with recurrent *L. monocytogenes* contamination demonstrated difficulties in collaborating with official control. Food business operator attitudes have been shown to affect their hygiene practices and rapport with authorities (Yapp & Fairman, 2006; Davies *et al.*, 2014; Läikkö-Roto & Nevas, 2014a). The prevention of *L. monocytogenes* contamination requires rigorous and constant efforts (Autio *et al.*, 1999; Lappi *et al.*, 2004b; Hu *et al.*, 2006), efforts that were suboptimal particularly in fish-processing plants exhibiting recurrent contamination. The understanding of food safety risks can help to value official control (Kettunen *et al.*, 2017a), but achieving compliance and reducing risks requires the creation of an attentive food safety culture (Griffith *et al.*, 2010; Griffith, 2010; Wu *et al.*, 2020) via appropriate management, resources, and infrastructure (Clayton *et al.*, 2002). Thus, the prevention of *L. monocytogenes* contamination must be a part of everyday operational practices at fish-processing plants, and efforts must be made to ensure that employees understand its importance (Hicks *et al.*, 2004).

6.3.3 Improving *L. monocytogenes* management by official food control measures

All planned annual inspections were not completed in most of the studied fish-processing plants. Additionally, not all fish-processing plant inspections were carried out during processing. These findings may imply inadequate or poorly organized resources. Indeed, insufficient personnel resources have been reported by several food control officials (Heikkilä *et al.*, 2016). The food safety legislation requires official control to be risk-based (EC no. 882/2004, EU no. 625/2017) and limited resources emphasize the importance of risk-based evaluation and prioritization of tasks. Judging by the frequent demands for correction and stringent time limits given to non-compliances estimated to predispose to *L. monocytogenes*, the inspectors seem to have focused on risky non-compliances. However, the relatively poor correction rates imply that control measures were not efficient in supporting and compelling the fish-processing plants to remove the violations observed.

Our results highlight the need to encourage the use of official control measures – both light and stringent – when necessary. *L. monocytogenes* occurrence increased with any type of recurring non-compliances and decreased, if non-compliances were followed by official control measures. Thus, addressing thoroughly both minor and major violations as part of official

control is important for the prevention of *L. monocytogenes* contamination. Based on the results of Study III, particular attention should be paid to non-compliances concerning cleanliness, maintenance, and hygienic handling of processing machines.

However, successful enforcement of official control measures is not always a simple task. Professionalism of the inspector and a negotiative approach motivate food business operators to perform corrections (Kettunen *et al.*, 2017a). Our observations regarding problems in collaboration speak on behalf of development of inspector communication skills in approaches that build trust and motivation. Acknowledging that human factors, such as emotions and social influences, compromise safe food handling (Thaivalappil *et al.*, 2018), inspectors may require additional interpersonal tools to advocate for improvements in food safety culture and nudge food business operators towards correction of non-compliances. Nonetheless, situations where coercive measures are necessary must also be seized by inspectors. According to our findings, most control measures were not enforced by coercive measures, even when the non-compliances recurred. While coercive processes are deemed effective (Kettunen *et al.*, 2015), some food inspectors use them scarcely for a variety of reasons (Lundén, 2013; Kettunen *et al.*, 2015; Luukkanen & Lundén, 2016; Kettunen *et al.*, 2017b). Since 2015, the structure of follow-up inspections has been developed in the Finnish food control system Oiva; processes of the reformed inspection system include the application of enforcement measures until non-compliances impairing or jeopardizing food safety have been corrected (Finnish Food Authority, 2019b). The potential of Oiva to improve the correction of non-compliances requires further investigation, for which our data and results provide a foundation. Promisingly, in a recent survey, 69% (n = 239) of food industry operators totally or somewhat agreed that the disclosure of inspection results introduced by Oiva advances the correction of non-compliances (Kaskela *et al.*, 2019).

Due to factors concerning the microbial ecology of *L. monocytogenes* and the characteristics of the products and production chain, gravad and cold-smoked fish products cannot be considered entirely safe from *L. monocytogenes* contamination. The possibility of reducing risks by modifying product composition and storage conditions may further be explored alongside pertinent contamination prevention. Consumers are left with the responsibility to store and handle these products according to instructions and recommendations. Moreover, people belonging to risk groups may need to avoid them entirely, as is currently recommended in Finland (Finnish Food Authority, 2019a). A recent Finnish risk assessment concluded that the risk for the elderly perseveres despite storage of these products at the recommended time and temperature (Pasonen *et al.*, 2019). Scientists, official authorities, and health care providers share the task of educating the general public on the threats of listeriosis. Finally, findings here clearly demonstrate the importance of stringent hygienic practices in the fish industry in preventing the contamination of facilities and products by *L. monocytogenes*.

7 CONCLUSIONS

The research described herein implements and develops new methodologies to study the stress tolerance of *L. monocytogenes* and the control of the bacterium in the fish industry. The results include novel discoveries of the strain variability and mechanisms of *L. monocytogenes* stress tolerance as well as the operational practices and official control procedures associated with *L. monocytogenes* contamination in fish-processing plants. The findings can be implemented in the investigation of detection and control measures for stress-tolerant *L. monocytogenes* strains and the development of prevention strategies for *L. monocytogenes* in the fish industry. Preventive measures can also be extrapolated to other food industries where *L. monocytogenes* poses a problem.

1. (i) At NaCl osmotic stress, large *L. monocytogenes* strain variability as well as serotype- and lineage-dependent phenotypes were discovered. *L. monocytogenes* strains of lineage I serotypes grew significantly better at NaCl 9.0% than strains of lineage II serotypes, which suggests that osmotic stress acclimation differs between *L. monocytogenes* lineages and sublineages.

   (ii) We highlighted the importance of handling all strains and data similarly and performing systematic error checking throughout data accumulation, inspection, and analysis of microbial growth studies on stress tolerance. The compiled data assembly and analysis protocol suits the investigation of strain variability of stress tolerance when relative differences between strains form the main question of interest.

2. A plasmid-borne ATP-dependent protease ClpL was found to contribute to the survival of *L. monocytogenes* at high temperature stress, which provided the first description of plasmid-mediated heat tolerance in *L. monocytogenes*. This increases the current understanding of accessory genetic mechanisms underlying strain variability of *L. monocytogenes* survival at heat treatments. Plasmid-borne ClpL may be relevant for heat tolerance also in other Gram-positive bacteria.

3. In the investigation of RTE vacuum-packaged fish products, *L. monocytogenes* contamination only occurred in sliced products and was significantly more common in gravad products than in cold-smoked products. Risk factors for *L. monocytogenes* product contamination included the following: processing machines, particularly slicing and skinning machines; staff movement between areas of low and high hygiene; infrequent periodic sanitation of the processing facilities; and lack

of periodic thorough sanitation of machinery. To strengthen *L. monocytogenes* prevention, hygienic routes require improvements, and periodic thorough sanitation practices should be more frequently implemented in several fish-processing plants. The attention of food safety management should be directed towards supporting staff commitment to continuous preventive measures.

4. Non-compliances estimated to predispose to *L. monocytogenes* occurred in many operations and processing machines of the studied fish-processing plants. Although official control had focused on risky operations, recurrence of non-compliances was common, indicating insufficient control methods and communication. Inefficacy of official control was shown to be associated with poor *L. monocytogenes* prevention. To reduce *L. monocytogenes* occurrence, timely correction of non-compliances – especially those pertaining to machinery, hygiene, and sanitation – should always be demanded. Improving the efficacy of official control should include the following: systematic re-inspection and enhanced supervision of time limits, which have since been developed in the "Oiva" system; improving cooperative communication; and increasing the use of coercive measures when needed.

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